COMPOSITIONS AND METHODS FOR MODULATING PHYSIOLOGY OF EPITHELIAL JUNCTIONAL ADHESION MOLECULES FOR ENHANCED MUCOSAL DELIVERY OF THERAPEUTIC COMPOUNDS

This claims priority under 35 U.S.C. §119 (e) to United States Provisional Application No. 60/392,512 filed June 28, 2002, the entire contents of which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

The teachings of all of the references cited in the present specification are incorporated in their entirety herein by reference.

A major disadvantage of drug administration by injection is that trained personnel are often required to administer the drug. For self-administered drugs, many patients are reluctant or unable to give themselves injections on a regular basis. Injection is also associated with increased risks of infection. Other disadvantages of drug injection include variability of delivery results between individuals, as well as unpredictable intensity and duration of drug action.

Despite these noted disadvantages, injection remains the only approved delivery mode for a large assemblage of important therapeutic compounds. These include conventional drugs, as well as a rapidly expanding list of peptide and protein biotherapeutics. Delivery of these compounds via alternate routes of administration, for example, oral, nasal and other mucosal routes, often yields variable results and adverse side effects, and fails to provide suitable bioavailabilty. For macromolecular species in particular, especially peptide and protein therapeutics, alternate routes of administration are limited by susceptibility to inactivation and poor absorption across mucosal barriers.

Mucosal administration of therapeutic compounds may offer certain advantages over injection and other modes of administration, for example in terms of convenience and speed of delivery, as well as by reducing or elimination compliance problems and side effects that attend delivery by injection. However, mucosal delivery of biologically active agents is limited by mucosal barrier functions and other factors. For these reasons, mucosal drug administration typically requires larger amounts of drug than administration by injection. Other therapeutic compounds,

including large molecule drugs, peptides and proteins, are often refractory to mucosal delivery.

The ability of drugs to permeate mucosal surfaces, unassisted by delivery-enhancing agents, appears to be related to a number of factors, including molecular size, lipid solubility, and ionization. Small molecules, less than about 300-1,000 daltons, are often capable of penetrating mucosal barriers, however, as molecular size increases, permeability decreases rapidly. Lipid-soluble compounds are generally more permeable through mucosal surfaces than are non-lipid-soluble molecules. Peptides and proteins are poorly lipid soluble, and hence exhibit poor absorption characteristics across mucosal surfaces.

In addition to their poor intrinsic permeability, large macromolecular drugs, including proteins and peptides, are often subject to limited diffusion, as well as lumenal and cellular enzymatic degradation and rapid clearance at mucosal sites. These mucosal sites generally serve as a first line of host defense against pathogens and other adverse environmental agents that come into contact with the mucosal surface. Mucosal tissues provide a substantial barrier to the free diffusion of macromolecules, while enzymatic activities present in mucosal secretions can severely limit the bioavailability of therapeutic agents, particularly peptides and proteins. At certain mucosal sites, such as the nasal mucosa, the typical residence time of proteins and other macromolecular species delivered is limited, e.g., to about 15-30 minutes or less, due to rapid mucociliary clearance.

In summary, previous attempts to successfully deliver therapeutic compounds, including small molecule drugs and protein therapeutics, via mucosal routes have suffered from a number of important and confounding deficiencies. These deficiencies point to a long-standing unmet need in the art for pharmaceutical formulations and methods of administering therapeutic compounds that are stable and well tolerated and that provide enhanced mucosal delivery, including to targeted tissues and physiological compartments such as central nervous system. More specifically, there is a need in the art for safe and reliable methods and compositions for mucosal delivery of therapeutic compounds for treatment of diseases and other adverse conditions in mammalian subjects. A related need exists for methods and compositions that will provide efficient delivery of macromolecular drugs via one or more mucosal routes in therapeutic amounts, which are fast acting, easily

administered and have limited adverse side effects such as mucosal irritation or tissue damage.

In relation to these needs, an especially challenging need persists in the art for methods and compositions to enhance mucosal delivery of biotherapeutic compounds that will overcome mucosal epithelial barrier mechanisms. Selective permeability of mucosal epithelia has heretofore presented major obstacles to mucosal delivery of therapeutic macromolecules, including biologically active peptides and proteins. Accordingly, there remains a substantial unmet need in the art for new methods and tools to facilitate mucosal delivery of biotherapeutic compounds. In particular, there is a compelling need in the art for new methods and formulations to facilitate mucosal delivery of biotherapeutic compounds that have heretofore proven refractory to delivery across mucosal barriers.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is the amino acid sequence of human JAM-1 wherein the extracelluar domain is underlined.

Figure 2 is the amino acid sequence of human JAM-2 wherein the extracelluar domain is underlined.

Figure 3 is the amino acid sequence of human JAM-3 wherein the extracelluar domain is underlined.

Figure 4 is the amino acid sequence of human claudin-1 wherein the extracelluar domains are underlined.

Figure 5 is the amino acid sequence of human claudin-2 wherein the extracelluar domains are underlined.

Figure 6 is the amino acid sequence of human claudin-3 wherein the extracelluar domains are underlined.

Figure 7 is the amino acid sequence of human claudin-4 wherein the extracelluar domains are underlined.

Figure 8 is the amino acid sequence of human claudin-5 wherein the extracelluar domains are underlined.

Figure 9 is the amino acid sequence of human claudin-6 wherein the extracelluar domains are underlined.

Figure 10 is the amino acid sequence of human claudin-7 wherein the extracelluar domains are underlined.

Figure 11 is the amino acid sequence of human claudin-8 wherein the extracelluar domains are underlined.

Figure 12 is the amino acid sequence of human claudin-9 wherein the extracelluar domains are underlined.

Figure 13 is the amino acid sequence of human claudin-10 wherein the extracelluar domains are underlined.

Figure 14 is the amino acid sequence of human claudin-2 wherein the extracelluar domains are underlined.

DESCRIPTION OF THE INVENTION

The instant invention satisfies the foregoing needs and fulfills additional objects and advantages by providing novel pharmaceutical compositions that include a biologically active agent and a permeabilizing agent effective to enhance mucosal delivery of the biologically active agent in a mammalian subject. The permeabilizing agent reversibly enhances mucosal epithelial paracellular transport, typically by modulating epithelial junctional structure and/or physiology at a mucosal epithelial surface in the subject. This effect typically involves inhibition by the permeabilizing agent of homotypic or heterotypic binding between epithelial membrane adhesive proteins of neighboring epithelial cells. Target proteins for this blockade of homotypic or heterotypic binding can be selected from various related junctional adhesion molecules (JAMs), occludins, or claudins.

Epithelial cells provide a crucial interface between the external environment and mucosal and submucosal tissues and extracellular compartments. One of the most important functions of mucosal epithelial cells is to determine and regulate mucosal permeability. In this context, epithelial cells create selective permeability barriers between different physiological compartments. Selective permeability is the result of regulated transport of molecules through the cytoplasm (the transcellular pathway) and the regulated permeability of the spaces between the cells (the paracellular pathway).

Intercellular junctions between epithelial cells are known to be involved in both the maintenance and regulation of the epithelial barrier function, and cell-cell adhesion. The tight junction (TJ) of epithelial and endothelial cells is a particularly

important cell-cell junction that regulates permeability of the paracellular pathway, and also divides the cell surface into apical and basolateral compartments. Tight junctions form continuous circumferential intercellular contacts between epithelial cells and create a regulated barrier to the paracellular movement of water, solutes, and immune cells. They also provide a second type of barrier that contributes to cell polarity by limiting exchange of membrane lipids between the apical and basolateral membrane domains.

Tight junctions are thought to be directly involved in barrier and fence functions of epithelial cells by creating an intercellular seal to generate a primary barrier against the diffusion of solutes through the paracellular pathway, and by acting as a boundary between the apical and basolateral plasma membrane domains to create and maintain cell polarity, respectively. Tight junctions are also implicated in the transmigration of leukocytes to reach inflammatory sites. In response to chemoattractants, leukocytes emigrate from the blood by crossing the endothelium and, in the case of mucosal infections, cross the inflamed epithelium. Transmigration occurs primarily along the paracellular rout and appears to be regulated via opening and closing of tight junctions in a highly coordinated and reversible manner.

Numerous proteins have been identified in association with TJs, including both integral and peripheral plasma membrane proteins. Current understanding of the complex structure and interactive functions of these proteins remains limited. Among the many proteins associated with epithelial junctions, several categories of trans-epithelial membrane proteins have been identified that may function in the physiological regulation of epithelial junctions. These include a number of "junctional adhesion molecules" (JAMs) and other TJ-associated molecules designated as occludins, claudins, and zonulin.

JAMs, occludin, and claudin extend into the paracellular space, and these proteins in particular have been contemplated as candidates for creating an epithelial barrier between adjacent epithelial cells and regulatable channels through epithelial cell layers. In one model, occludin, claudin, and JAM have been proposed to interact as homophilic binding partners to create a regulated barrier to paracellular movement of water, solutes, and immune cells between epithelial cells.

A cDNA encoding murine junctional adhesion molecule-1 (JAM-1) has been cloned and corresponds to a predicted type I transmembrane protein (comprising a single transmembrane domain) with a molecular weight of approximately 32-kD

(Williams, et al., Molecular Immunology 36: 1175-1188 1999; Gupta, et al., IUBMB Life, 50: 51-56, 2000; Ozaki, et al., J. Immunol. 163: 553-557, 1999; Martin-Padura, et al., J Cell Biol 142: 117-127, 1998). The extracellular segment of the molecule comprises two Ig-like domains described as an amino terminal "VH-type" and a carboxy-terminal "C2-type" carboxy-terminal β-sandwich fold (Bazzoni et al., Microcirculation 8:143-152, 2001). Murine JAM-1 also contains two sites for N-glycosylation, and a cytoplasmic domain. The JAM-1 protein is a member of the immunoglobulin (Ig) superfamily and localizes to tight junctions of both epithelial and endothelial cells. Ultrastructural studies indicate that JAM-1 is limited to the membrane regions containing fibrils of occludin and claudin.

Transfection of a murine JAM-1-encoding cDNA into CHO cells leads to localization of the JAM-1 protein at cell-cell contacts, which only occurs in confluent monolayers when neighboring cells express JAM. In mixed cultures, where JAM transfectants are in contact with control transfectants, the protein remains diffuse--suggesting that JAM clustering is due to homophyllic interaction (Martin-Padura, et al., J Cell Biol 142: 117-127, 1998).

Experimental evidence suggests that JAM-1 can mediate homotypic adhesion and influence monocyte transmigration via heterotypic adhesive and de-adhesive interactions. A monoclonal antibody against murine JAM-1 inhibits transmigration of leukocytes across endothelial cells and in an *in vivo* model of skin inflammatory reaction (Martin-Padura, et al., J Cell Biol 142: 117-127, 1998). Anti-murine JAM-1 antibodies also inhibit accumulation of leukocytes in the cerebrospinal fluid in cytokine-induced meningitis. It is unknown how these effects are mediated. In one model, the antibodies may inhibit a heterotypic interaction between JAM-1 and a leukocyte receptor (see, e.g., Del Maschio et al., J. Exp. Med. 190:1351-1356, 1999). Alternatively, the anti-JAM-1 antibodies may stabilize a homophilic JAM-mediated interaction between neighboring cells and thereby inhibit dissociation of the junctional complex (see, e.g., Balda et al., Cell Devel. Biol. 11: 281-289, 2000).

One model for JAM-1 activity proposes that an extracellular domain of JAM-1 is involved in intercellular adhesive interactions. Formation of JAM-1 dimers is thought to be due to stable and noncovalent interactions. Dissociation of JAM-1 dimers into monomeric subunits is reported at high ionic strength and acidic pH. In this general model, JAM-1 dimers are hypothesized to act as building blocks for

JAM-1-dependent homophilic adhesion. In particular, JAM-1 may dimerize in cis-interactions yielding parallel homodimers positioned at one cell surface, and the cis-dimerization might expose an interface available for homophilic adhesive interactions between JAM-1 molecules on opposing cell surfaces. This model could account for homotypic adhesion between adjoining cells within confluent endothelial or epithelial monolayers. In addition, JAM-1 dimers expressed on transmigrating leukocytes are proposed to interact with JAM-1 dimers expressed on endothelial cells, thus accounting for the adhesion and de-adhesion events that occur during leukocyte transendothelial migration. (Dejana, et al., Throb. Haemost. 86: 308-315, 2001)

A crystal structure of a recombinant soluble form of murine JAM-1 protein (a truncated extracellular region of the molecule designated "rsJAM") has been described. (Kostrewa, et al., EMBO J., 20: 4391-4398, 2001). The rsJAM construct is proposed to consist of two immunoglobulin-like domains connected by a conformationally restrained short linker. Two JAM molecules reportedly form a U-shaped dimer by complementary interactions including two salt bridges between respective rsJAM constructs. The report further identifies a central tri-peptide of rsJAM (Arg58-Val59-Glu60) that corresponds to a proposed conservative "motif for dimerization". This conservative motif, "R(V,I,L)E", is suggested to mediate formation of rsJAM dimers in solution. The R(V,I,L)E is motif, as well as flanking residues Trp61, Lys62, Cys73, and Tyr74, are noted by the authors to be conserved in published sequences of murine, bovine and human JAM-1. Moreover, the sequence R(V,I,L)E is noted to also be conserved in Jam-2 and JAM-3.

Studies of mutant rsJAM that have been engineered to introduce a disruptive point mutation in the proposed dimerization motif (Glu60Arg), suggest that the mutation blocks homotypic aggregation of rsJAM (Kostrewa, et al., EMBO J., 20: 4391-4398, 2001). Based on these mutant studies and on analysis of crystal packing of rsJAM, a more detailed model for homophilic adhesion of JAM has been proposed. In this model, JAM cis-dimers are believed to form on the cell surface, and the cis-dimerization is proposed to be a necessary precursor to adhesive trans-interactions between dimerized JAM molecules on opposing cell surfaces.

Additional studies have reported the identification of human, rat, and bovine counterparts of murine JAM-1 (see, e.g., Liu et al., J. Cell. Sci. 113:2363-2374, 2000; Ozaki et al., J. Immunol. 163:553-557, 1999; Wiliams et al., Mol. Immunol. 36:1175-1188, 1999; and Sobocka et al., Blood 95: 2600-2609, 2000). These different JAM-1

homologues exhibits between 68%-75% overall amino acid identity with the murine JAM-1 protein sequence. There is said to be a "[s]triking sequence similarity in the transmembrane and cytoplasmic tail regions in particular—suggesting an important and conserved function for these proteins perhaps involving protein interactions at the cytoplasmic interface (see, e.g., Williams et al. (Mol. Immunol. 36:1175-1188, 1999). There is also noted to be general structural conservation among these different JAM-1 homologs in terms of their extracellular structure—which each exhibit amino-terminal and carboxy-terminal β-sandwich folds proposed to represent tandem V_H- and C_2-type Ig-like domains (see, e.g., Dejana, et al. (Throb. Haemost. 86: 308-315, 2001).

The putative extracellular domain of human JAM-1 was recently expressed as a fusion protein to generate anti-human JAM-1 antibodies that inhibited transepithelial resistance recovery (TER) in T84 cell monolayers after tight junction disruption mediated by transient calcium depletion (Liu et al., J. Cell. Sci. 113:2363-2374, 2000). In particular, the anti-JAM antibodies inhibit JAM-1 and occludin redistribution to TJs following calcium mediated disruption. However, these authors report that purified recombinant human JAM-1 containing the extracellular domain does not inhibit TER after tight junction disruption, contrary to published results for murine JAM-1. On this basis it is considered that the data may not support a model of extracellular homo- or heterotypic interaction mediated by the human JAM-1 extracellular domain. In another study investigating the structure/function of human JAM-1, Williams et al. (Mol. Immunol. 36:1175-1188, 1999) report that both murine and junman JM Fc chimeras and transfected COS cells failed to show homotypic adhesion for the protein *in vitro*—suggesting that "firm adhesion may not be the function of this molecule *in vivo*." In a separate study, Liang et al. (Am. J. Physiol. 279:1733-1743, 2000,) report that a recombinant soluble form of human JAM-1 inhibits recovery of TER following trypsin-EDTA disruption of TJs.

Additional molecules have been identified with apparent homology to JAM-1. A recently identified JAM2 cDNA corresponds to a predicted 34-kD type I integral membrane protein featuring two Ig-like folds and three N-linked glycosylation sites in the extracellular domain. A single protein kinase C phophorylation consensus site and a PDZ-binding motif are predicted in the short cytoplasmic tail. Northern blot analysis suggests that JAM2 is preferentially expressed in the heart (Cunningham et al., J. Biol. Chem., 275: 34750-34756, 2000). In a related International Publication

(WO 01/14404), Cunningham teaches that JAM-2, unlike JAM-1, does not show expression in peripheral blood leukocytes, and that it is unknown whether JAM-2 functions in homotypic interactions. Cunningham speculates that it may be possible to use a fusion between the JAM-2 extracellular sequence and the Fc region of mouse/human IgG to: screen for a JAM-2 ligand; screen for small molecule inhibitor of JAM-2 heterotypic interactions; or to neutralize JAM-2 function in either heterotypic or homotypic interactions.

Another JAM family member, designated "Vascular endothelial junction-associated molecule" (VE-JAM), contains two extracellular immunoglobulin-like domains, a transmembrane domain, and a relatively short cytoplasmic tail. VE-JAM is principally localized to intercellular boundaries of endothelial cells (Palmeri, et al., J. Biol. Chem., 275: 19139-19145, 2000,). VE-JAM is highly expressed highly by endothelial cells of venules, and is also expressed by endothelia of other vessels. Another reported JAM family member, JAM-3, has a predicted amino acid sequence that displays 36% and 32% identity, respectively, to JAM-2 and JAM-1. JAM-3 shows widespread tissue expression with higher levels apparent in the kidney, brain, and placenta. At the cellular level, JAM-3 transcript is expressed within endothelial cells. JAM-3 and JAM-2 have been reported to be binding partners. In particular, the JAM-3 ectodomain reportedly binds to JAM2-Fc. JAM-3 protein is up-regulated on peripheral blood lymphocytes following activation. (Pia Arrate, et al., J. Biol. Chem., 276: 45826-45832, 2001).

Another proposed trans-membrane adhesive protein involved in epithelial tight junction regulation is Occludin. Occludin is an approximately 65-kD type II transmembrane protein composed of four transmembrane domains, two extracellular loops, and a large C-terminal cytosolic domain (Furuse et al., J. Cell Biol. 123:1777 - 1788, 1993; Furuse et al., J Cell Biol 127:1617-1626, 1994). This topology has been confirmed by antibody accessibility studies (Van Itallie, and Anderson, J. Cell. Sci. 110: 1113-1121,1997,). The extracellular loops are chemically distinct. The first extracellular loop contains approximately 65% tyrosine and glycine residues. Although the presence of alternating tyrosine and glycine residues is conserved in all five occludin homologs from different animal species presently cloned, the functional significance of this particular sequence is unclear (Fujimoto. J. Cell. Sci. 108:3443 - 3449, 1995).

Occludin is proposed to be a Ca^{2+}-independent intercellular adhesion molecule. When expressed in fibroblasts lacking endogenous occludin, it confers adhesiveness in proportion to the level of occludin expressed. This artificially conferred adhesiveness is reportedly blocked by peptides corresponding to either of the two extracellular loops of occludin. Nonetheless, it remains to be determined whether occludin is a homotypic adhesion molecule or has a yet unidentified counter-receptor. (Chen et al., J. Cell Biol. 138:891-899, 1997; Fanning et al., J. Am. Soc. Nephrol. 10:1337-1345, 1999). Occludin is also capable of lateral oligomerization through side-to-side associations, perhaps within the membrane bilayer. Given its adhesive properties, occludin might create a paracellular barrier by polymerizing laterally in the membrane to create a continuous line of adhesion between cells.

When observed by immuno-freeze fracture electron microscopy, occludin is concentrated directly within the tight junction fibrils (Fujimoto. J Cell Sci 108:3443 - 3449, 1995,). Immunofluorescence localization reveals an additional minor pool of occludin along the lateral membrane that is more easily extracted in nonionic detergents, less phosphorylated, and not assembled into fibrils (Sakakibara, et al., J Cell Biol 137:1393 -1401, 1997; Cordenonsi, et al., J Cell Sci 110:3131 -3139, 1997). Conceivably, the lateral pool represents a reservoir of subunits available for dynamic regulated expansion of junctional complexity.

Two additional integral membrane proteins of the tight junction, claudin-1 and claudin-2, were identified by direct biochemical fractionation of junction-enriched membranes from chicken liver (Furuse, et al., J Cell Biol 141 : 1539-1550,1998). Claudin-1 and claudin-2 were found to copurify with occludin as a broad approximately 22-kD gel band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The deduced sequences of two closely related proteins cloned from a mouse cDNA library predict four transmembrane helices, two short extracellular loops, and short cytoplasmic N- and C-termini. Despite topologies similar to that of occludin, they share no sequence homology. Subsequently, six more claudin gene products (claudin-3 through claudin-8) have been cloned and have been shown to localize within tight junction fibrils, as determined by immunogold freeze fracture labeling (Morita et al., Proc Natl Acad Sci USA 96 : 511-516, 1999). Given that a barrier remains in the absence of occludin, claudin-1 through claudin-8 have been considered as candidates for the primary seal-forming elements of the extracellular space. Consistent with this role, when either claudin-1 or -2 is expressed in

fibroblasts, these proteins are capable of assembling into long branching fibrils reminiscent of their organization in the tight junction of epithelial cells. In contrast, occludin has a limited ability to self-organize into fibrils in transfected fibroblasts, but will join the fibrils when claudin is cotransfected (Furuse, et al., J Cell Biol 143:391 - 401, 1998).

Other cytoplasmic proteins that have been localized to epithelial junctions include zonulin, symplekin, cingulin, and 7H6. Zonulins reportedly are cytoplasmic proteins that bind the cytoplasmic tail of occludin. Representing this family of proteins are "ZO-1, ZO-2, and ZO-3". Zonulin is postulated to be a human protein analogue of the *Vibrio cholerae* derived zonula occludens toxin (ZOT).

Zonulin likely plays a role in tight junction regulation during developmental, physiological, and pathological processes--including tissue morphogenesis, movement of fluid, macromolecules and leukocytes between the intestinal lumen and the interstitium, and inflammatory/autoimmune disorders (see, e.g., Wang, et al., J. Cell Sci., 113:4435-40, 2000; Fasano, et al., Lancet 355:1518-9, 2000; Fasano, Ann. N.Y. Acad. Sci., 915: 214-222, 2000). Zonulin expression increased in intestinal tissues during the acute phase of coeliac disease, a clinical condition in which tight junctions are opened and permeability is increased. Zonulin induces tight junction disassembly and a subsequent increase in intestinal permeability in non-human primate intestinal epithelia *in vitro*.

Comparison of amino acids in the active *V. cholerae* ZOT fragment and human zonulin identified a putative receptor binding domain within the N-terminal region of the two proteins. The ZOT biologically active domain increases intestinal permeability by interacting with a mammalian cell receptor with subsequent activation of intracellular signaling leading to the disassembly of the intercellular tight junction. The ZOT biologically active domain has been localized toward the carboxyl terminus of the protein and coincides with the predicted cleavage product generated by *V. cholerae*. This domain shares a putative receptor-binding motif with zonulin, the ZOT mammalian analogue. Amino acid comparison between the ZOT active fragment and zonulin, combined with site-directed mutagenesis experiments, suggest an octapeptide receptor-binding domain toward the amino terminus of processed ZOT and the amino terminus of zonulin. (Di Pierro, et al., J. Biol. Chem., 276: 19160-19165, 2001). ZO-1 reportedly binds actin, AF-6, ZO-associated kinase (ZAK), fodrin, and α-catenin.

In more detailed embodiments of the invention, the permeabilizing agent is a peptide or peptide analog or mimetic. Exemplary permeabilizing peptides comprise from about 4-25 contiguous amino acids of an extracellular domain of a mammalian JAM-1, JAM-2, or JAM-3 protein. Alternatively, the permeabilizing peptide may comprise from about 6-15 contiguous amino acids of an extracellular domain of a mammalian JAM-1, JAM-2, or JAM-3 protein. In additional embodiments, the permeabilizing peptide comprises from about 4-25 contiguous amino acids of an extracellular domain of a mammalian JAM-1, JAM-2, or JAM-3 protein, or a sequence of amino acids that exhibits at least 85% amino acid identity with a corresponding reference sequence of 4-25 contiguous amino acids of an extracellular domain of a mammalian JAM-1, JAM-2, or JAM-3 protein. In certain embodiments, the amino acid sequence of the permeabilizing peptide exhibits one or more amino acid substitutions, insertions, or deletions compared to the corresponding reference sequence of the mammalian JAM-1, JAM-2, or JAM-3 protein. For example, the permeabilizing peptide may exhibit one or more conservative amino acid substitutions compared to a corresponding reference sequence of a mammalian JAM-1, JAM-2, or JAM-3 protein. Such functional peptide analogs or variants may, for instance, have one or more amino acid mutations in comparison to a corresponding wild-type sequence of the same human JAM protein (e.g., human JAM-1), wherein the mutation(s) correspond to a divergent amino acid residue or sequence identified in a different human JAM protein (e.g., human JAM-2 or JAM-3) or in a homologous JAM protein found in a different species (e.g. murine, rat, or bovine JAM-1, JAM-2 or JAM-3 protein).

In more detailed embodiments, the methods and compositions of the invention incorporate a permeabilizing peptide that is between about 4-25 amino acids in length, and includes one or more contiguous sequence elements selected from: V R (I, V, A) P (SEQ ID NO: 1); (V, A, I) K L (S, T) C A Y (SEQ ID NO: 2); or E D (T, S) G T Y (T,R) C (M, E) (SEQ ID NO: 3). In one such embodiment, the peptide will include a conservative sequence motif V R (I, V, A) P (SEQ ID NO: 1), wherein the third position of the motif may be represented by one of the alternative amino acid residues I, V, or A. In another such embodiment, the peptide will include a conservative sequence motif (V, A, I) K L (S, T) C A Y (SEQ ID NO: 2), wherein the first position of the motif may be represented by one of the alternative amino acid residues V, A, or

I, and the fourth position of the motif may be represented by one of the alternative amino acid residues S or T. In yet another such embodiment, the peptide will include a conservative sequence motif E D (T, S) G T Y (T,R) C (M, E) (SEQ ID NO: 3), wherein the third position of the motif may be represented by one of the alternative amino acid residues T or S, the seventh position of the motif may be represented by one of the alternative amino acid residues T or R, and the ninth position of the motif may be represented by one of the alternative residues M or E. In exemplary embodiments, the permeabilizing peptide is between about 4-25 amino acids in length and includes one or more contiguous sequence elements selected from wild-type human JAM-1 peptide sequences VRIP (SEQ ID NO: 4), VKLSCAY (SEQ ID NO: 5), TGITFKSVT (SEQ ID NO: 6), ITAS (SEQ ID NO: 7), SVTR (SEQ ID NO: 8), EDTGTYTCM (SEQ ID NO: 9), and/or GFSSPRVEW (SEQ ID NO: 10).

Within additional aspects of the invention, pharmaceutical compositions and methods are provided which employ a permeabilizing peptide comprising from about 4-25 contiguous amino acids of an extracellular domain of a mammalian occludin protein. In alternate embodiments, the permeabilizing peptide comprises from about 6-15 contiguous amino acids of an extracellular domain of a mammalian occludin protein. In certain aspects, the permeabilizing peptide comprises from about 4-25 contiguous amino acids of an extracellular domain of a mammalian occludin protein or comprises an amino acid sequence that exhibits at least 85% amino acid identity with a corresponding reference sequence of 4-25 contiguous amino acids of an extracellular domain of a mammalian occludin protein. In exemplary embodiments, the permeabilizing peptide exhibits one or more amino acid substitutions, insertions, or deletions compared to a corresponding reference sequence of the mammalian occludin protein. Often, such peptide "analogs" will exhibit one or more conservative amino acid substitutions compared to the corresponding reference sequence of the mammalian occludin protein. In related embodiments, the permeabilizing peptide is a human occludin peptide and the amino acid sequence of the permeabilizing peptide exhibits one or more amino acid mutations in comparison to a corresponding wild-type sequence of the same human occludin protein, wherein the mutation(s) correspond to a structural feature (e.g., a divergent, aligned residue or sequence of residues) identified in a different human occludin protein or a homologous occludin protein found in a different species.

Within other aspects of the invention, pharmaceutical compositions and methods are provided which employ a permeabilizing peptide comprising from about 4-25 contiguous amino acids of an extracellular domain of a mammalian claudin protein. In alternate embodiments, the permeabilizing peptide comprises from about 6-15 contiguous amino acids of an extracellular domain of a mammalian claudin protein. In certain aspects, the permeabilizing peptide comprises from about 4-25 contiguous amino acids of an extracellular domain of a mammalian claudin protein or comprises an amino acid sequence that exhibits at least 85% amino acid identity with a corresponding reference sequence of 4-25 contiguous amino acids of an extracellular domain of a mammalian claudin protein. In exemplary embodiments, the permeabilizing peptide exhibits one or more amino acid substitutions, insertions, or deletions compared to a corresponding reference sequence of the mammalian claudin protein. Often, such peptide "analogs" will exhibit one or more conservative amino acid substitutions compared to the corresponding reference sequence of the mammalian claudin protein. In related embodiments, the permeabilizing peptide is a human claudin peptide and the amino acid sequence of the permeabilizing peptide exhibits one or more amino acid mutations in comparison to a corresponding wild-type sequence of the same human claudin protein, wherein the mutation(s) correspond to a structural feature (e.g., a divergent, aligned residue or sequence of residues) identified in a different human claudin protein or a homologous claudin protein found in a different species.

In related aspects of the invention, the pharmaceutical composition includes the permeabilizing agent and one or more biologically active agent(s) selected from a small molecule drug, a peptide, a protein, and a vaccine agent. In more detailed aspects, the biologically active agent(s) is/are selected from an opiod, opiod antagonist, corticosterone, anti-inflammatory, androgen, estrogen, progestin, muscle relaxant, vasodilator, antihistamine, histamine receptor site blocking agent, antitussive, antiepileptic, anti-fungal agent, antibacterial agent, cancer therapeutic agent, antioxidant, antiarrhythmic agents, antihypertensive agent, monoclonal or polyclonal antibody, anti-sense oligonucleotide, and/or an RNA, DNA or viral vector comprising a gene encoding a therapeutic peptide or protein. In other detailed aspects, the biologically active agent(s) is/are selected from a therapeutic protein or peptide, for example a protein or active peptide fragment or fusion of tissue plasminogen activator (TPA), epidermal growth factor (EGF), fibroblast growth

factor (FGF-acidic or basic), platelet derived growth factor (PDGF), transforming growth factor (TGF-alpha or beta), vasoactive intestinal peptide, tumor necrosis factor (TGF), hypothalmic releasing factors, prolactin, thyroid stimulating hormone (TSH), adrenocorticotropic hormone (ACTH), parathyroid hormone (PTH), follicle stimulating hormone (FSF), luteinizing hormone releasing (LHRH), endorphins, glucagon, calcitonin, oxytocin, carbetocin, aldoetecone, enkaphalins, somatostin, somatotropin, somatomedin, gonadotrophin, estrogen, progesterone, testosterone, alpha-melanocyte stimulating hormone, non-naturally occurring opiods, lidocaine, ketoprofen, sufentainil, terbutaline, droperidol, scopolamine, gonadorelin, ciclopirox, olamine, buspirone, calcitonin, cromolyn sodium or midazolam, cyclosporin, lisinopril, captopril, delapril, cimetidine, ranitidine, famotidine, superoxide dismutase, asparaginase, arginase, arginine deaminease, adenosine deaminase ribonuclease, trypsin, chemotrypsin, papain, bombesin, substance P, vasopressin, alpha-globulins, transferrin, fibrinogen, beta-lipoproteins, beta-globulins, prothrombin, ceruloplasmin, alpha$_2$-glycoproteins, alpha$_2$-globulins, fetuin, alpha-lipoproteins, alpha-globulins, albumin, and/or prealbumin.

In yet additional embodiments, the invention provides methods and pharmaceutical compositions which employ a permeabilizing agent as described above, such as a permeabilizing peptide, and one or more therapeutic protein(s) or peptide(s) that is/are effective as a hematopoietic agent, cytokine agent, antiinfective agent, antidementia agent, antiviral agent, antitumoral agent, antipyretic agent, analgesic agent, antiinflammatory agent, antiulcer agent, antiallergic agent, antidepressant agent, psychotropic agent, cardiotonic agent, antiarrythmic agent, vasodilator agent, antihypertensive agent, antidiabetic agent, anticoagulant agent, cholesterol-lowering agent, hormone agent, anti-osteoporosis agent, antibiotic agent, vaccine agent, and/or bacterial toxoid.

In certain embodiments of the invention, a biologically active agent and a permeabilizing agent as described above are administered in combination with one or more mucosal delivery-enhancing agent(s). In more detailed embodiments, the mucosal delivery-enhancing agent(s) is/are selected from:

(a) an aggregation inhibitory agent;

(b) a charge modifying agent;

(c) a pH control agent;

(d) a degradative enzyme inhibitory agent;

(e) a mucolytic or mucus clearing agent;

(f) a ciliostatic agent;

(g) a membrane penetration-enhancing agent selected from (i) a surfactant, (ii) a bile salt, (ii) a phospholipid additive, mixed micelle, liposome, or carrier, (iii) an alcohol, (iv) an enamine, (v) an NO donor compound, (vi) a long-chain amphipathic molecule (vii) a small hydrophobic penetration enhancer; (viii) sodium or a salicylic acid derivative; (ix) a glycerol ester of acetoacetic acid (x) a cyclodextrin or beta-cyclodextrin derivative, (xi) a medium-chain fatty acid, (xii) a chelating agent, (xiii) an amino acid or salt thereof, (xiv) an N-acetylamino acid or salt thereof, (xv) an enzyme degradative to a selected membrane component, (ix) an inhibitor of fatty acid synthesis, or (x) an inhibitor of cholesterol synthesis; or (xi) any combination of the membrane penetration enhancing agents recited in (i)-(x);

(h) a second modulatory agent of epithelial junction physiology;

(i) a vasodilator agent;

(j) a selective transport-enhancing agent; and

(k) a stabilizing delivery vehicle, carrier, support or complex-forming species with which the biologically active agent is effectively combined, associated, contained, encapsulated or bound resulting in stabilization of the active agent for enhanced mucosal delivery, wherein said one or more mucosal delivery-enhancing agents comprises any one or any combination of two or more of said mucosal delivery-enhancing agents recited in (a)-(k), and wherein the formulation of said biologically active agent with said mucosal delivery-enhancing agents provides for increased bioavailability of the biologically active agent delivered to a mucosal surface of a mammalian subject.

In more detailed embodiments of the inventions, the pharmaceutical compositions noted above are formulated for intranasal administration. In exemplary embodiments, the formulations are provided as an intranasal spray or powder. To enhance intranasal administration, these formulations may combine the biologically active agent and permeabilizing agent with one or more intranasal delivery-enhancing agents selected from:

(a) an aggregation inhibitory agent;

(b) a charge modifying agent;

(c) a pH control agent;

(d) a degradative enzyme inhibitory agent;

(e) a mucolytic or mucus clearing agent;

(f) a ciliostatic agent;

(g) a membrane penetration-enhancing agent selected from (i) a surfactant, (ii) a bile salt, (ii) a phospholipid additive, mixed micelle, liposome, or carrier, (iii) an alcohol, (iv) an enamine, (v) an NO donor compound, (vi) a long-chain amphipathic molecule (vii) a small hydrophobic penetration enhancer; (viii) sodium or a salicylic acid derivative; (ix) a glycerol ester of acetoacetic acid (x) a cyclodextrin or beta-cyclodextrin derivative, (xi) a medium-chain fatty acid, (xii) a chelating agent, (xiii) an amino acid or salt thereof, (xiv) an N-acetylamino acid or salt thereof, (xv) an enzyme degradative to a selected membrane component, (ix) an inhibitor of fatty acid synthesis, or (x) an inhibitor of cholesterol synthesis; or (xi) any combination of the membrane penetration enhancing agents recited in (i)-(x);

(h) a second modulatory agent of epithelial junction physiology;

(i) a vasodilator agent;

(j) a selective transport-enhancing agent; and

(k) a stabilizing delivery vehicle, carrier, support or complex-forming species with which the biologically active agent is effectively combined, associated, contained, encapsulated or bound resulting in stabilization of the active agent for enhanced intranasal delivery, wherein said one or more intranasal delivery-enhancing agents comprises any one or combination of two or more of said intranasal delivery-enhancing agents recited in (a)-(k), and wherein the formulation of said biologically active agent with said one or more intranasal delivery-enhancing agents provides for increased bioavailability of the biologically active agent delivered to a nasal mucosal surface of a mammalian subject.

In other related aspects of the invention, the pharmaceutical compositions comprising a permeabilizing agent, e.g., a permeabilizing peptide, and a biologically active agent are effective following mucosal administration to a mammalian subject to yield enhanced bioavailability of the therapeutic compound, for example by yielding a peak concentration (C_{max}) of the biologically active agent in a blood plasma or cerebral spinal fluid (CNS) of the subject that is about 25% or greater as compared to a peak concentration of the biologically active agent following intramuscular injection of an equivalent concentration or dose of the active agent to the subject. In certain embodiments, the pharmaceutical composition following mucosal administration yields a peak concentration (C_{max}) of the biologically active agent in the blood plasma

or CNS of the subject that is about 50% or greater than the peak concentration of the biologically active agent in the blood plasma or CNS following intramuscular injection of an equivalent concentration or dose of the active agent.

In alternate embodiments of the invention, the pharmaceutical compositions comprising a permeabilizing agent and a biologically active agent are effective following mucosal administration to yield enhanced bioavailability by yielding an area under concentration curve (AUC) of the biologically active agent in a blood plasma or cerebral spinal fluid (CNS) of the subject that is about 25% or greater compared to an AUC of the biologically active agent in blood plasma or CNS following intramuscular injection of an equivalent concentration or dose of the active agent to the subject. In certain embodiments, the pharmaceutical compositions yield an area under concentration curve (AUC) of the biologically active agent in a blood plasma or cerebral spinal fluid (CNS) of the subject that is about 50% or greater compared to an AUC of the biologically active agent in blood plasma or CNS following intramuscular injection of an equivalent concentration or dose of the active agent to the subject.

In additional embodiments of the invention, the pharmaceutical compositions comprising a permeabilizing agent and a biologically active agent are effective following mucosal administration to yield enhanced bioavailability by yielding a time to maximal plasma concentration (t_{max}) of said biologically active agent in a blood plasma or cerebral spinal fluid (CNS) of the subject between about 0.1 to 1.0 hours. In certain embodiments, the compositions yield a time to maximal plasma concentration (t_{max}) of the biologically active agent in a blood plasma or cerebral spinal fluid (CNS) of the subject between about 0.2 to 0.5 hours.

In other embodiments of the invention, the pharmaceutical compositions comprising a permeabilizing agent and a biologically active agent are effective following mucosal administration to yield enhanced bioavailability of the active agent in the CNS, for example by yielding a peak concentration of the biologically active agent in a CNS tissue or fluid of the subject that is 10% or greater compared to a peak concentration of the biologically active agent in a blood plasma of the subject (e.g., wherein the CNS and plasma concentration is measured contemporaneously in the same subject following the mucosal administration). In certain embodiments, compositions of the invention yield a peak concentration of the biologically active

agent in a CNS tissue or fluid of the subject that is 20%, 40%, or greater compared to a peak concentration of the active agent in a blood plasma of the subject.

In more detailed aspects of the invention, the pharmaceutical compositions and methods employing the permeabilizing agent and biologically active agent are effective for treating sexual dysfunction in mammalian subjects. In certain embodiments, the compositions and methods are effective for treating male and/or female sexual dysfunction. In exemplary embodiments, the compositions and methods are effective to treat male and/or female erectile dysfunction, e.g., by stimulating engorgement of erectile tissues in male and/or female subjects. In related embodiments, the methods and compositions are effective to enhance male and/or female sexual desire, competence for completing intercourse, and or ability to achieve a sexual stimulatory response, including orgasm. In more detailed aspects, compositions and methods of the invention for treating sexual dysfunction may employ a dopamine receptor agonist, or pharmaceutically acceptable salt or derivative thereof, as the biologically active agent. For example, the biologically active agent may be apomorphine or a pharmaceutically acceptable salt or derivative thereof. These methods and compositions are effective for treatment and prevention of diseases or conditions amenable to treatment by therapeutic administration of a dopamine receptor agonist, for example by stimulating engorgement of a male or female erectile tissue, and/or enhancing neural stimulation potential of said erectile tissue, by enhancing sexual desire, or by increasing the subject's ability to reach orgasm during sexual stimulation. In additional embodiments of the invention, a dopamine receptor agonist may be administered according to the compositions and methods herein to effectively treat or prevent symptoms of Parkinson's disease in mammalian subjects.

In certain exemplary embodiments of the invention, the biologically active agent(s) is/are selected from interferon-α, interferon-β, human growth hormone (HGH), insulin, heparin, nerve growth factor (NGF), erythropoietin (EPO), adrenocorticotropin hormone (ACTH), amyloid peptide, beta-sheet blocking peptide, natriuretic peptide, ketoprofen, and oleamide, oxoytocin, carbotocin, 5-hydroxytryptophan (seretonin) and the compositions and methods are effective for treatment of diseases, conditions and disorders amenable to treatment by mucosal administration of the foregoing active agent(s).

The methods of the invention for treating or preventing a disease or condition in a mammalian subject amenable to treatment by therapeutic administration of one or more of the biologically active agents identified herein generally comprise coordinately, mucosally administering to said subject a pharmaceutical formulation comprising a biologically active agent (e.g., a dopamine receptor agonist) and an effective amount of a permeabilizing agent (e.g., a permeabilizing peptide), as described above, to enhance mucosal delivery of the biologically active agent. Coordinate administration of the permeabilizing agent reversibly enhances mucosal epithelial paracellular transport by modulating epithelial junctional structure and/or physiology in a target mucosal epithelium of the subject. Typically, the permeabilizing agent effectively inhibits homotypic or heterotypic binding of an epithelial membrane adhesive protein selected from a junctional adhesion molecule (JAM), occludin, or claudin. In certain embodiments, the step(s) of coordinate mucosal administration involves delivery of the permeabilizing agent before, after, or simultaneous with (e.g., in a combinatorial formulation) delivery of the biologically active agent to a mucosal surface of the subject. In more detailed embodiments, the permeabilizing agent is coordinately administered with the biologically active agent to a nasal mucosal surface of said subject, for example in a combinatorial or separate nasal spray, gel or powder formulation(s). In exemplary embodiments, the permeabilizing agent is a permeabilizing peptide administered coordinately with the biologically active agent to yield enhanced mucosal epithelial paracellular transport of the biologically active agent. In certain exemplary embodiments, the permeabilizing peptide comprises from about 4-25, or about 6-15, contiguous amino acids of an extracellular domain of a mammalian JAM, occludin or claudin protein as described above, or a comparable length peptide that exhibits at least 85% amino acid identity with a corresponding reference sequence of an extracellular domain of a mammalian JAM, occludin or claudin protein.

Within additional coordinate administration methods of the invention, the biologically active agent and permeabilizing agent are administered in combination with one or more mucosal delivery-enhancing agents selected from:

(a) an aggregation inhibitory agent;

(b) a charge modifying agent;

(c) a pH control agent;

(d) a degradative enzyme inhibitory agent;

(e) a mucolytic or mucus clearing agent;

(f) a ciliostatic agent;

(g) a membrane penetration-enhancing agent selected from (i) a surfactant, (ii) a bile salt, (ii) a phospholipid additive, mixed micelle, liposome, or carrier, (iii) an alcohol, (iv) an enamine, (v) an NO donor compound, (vi) a long-chain amphipathic molecule (vii) a small hydrophobic penetration enhancer; (viii) sodium or a salicylic acid derivative; (ix) a glycerol ester of acetoacetic acid (x) a cyclodextrin or beta-cyclodextrin derivative, (xi) a medium-chain fatty acid, (xii) a chelating agent, (xiii) an amino acid or salt thereof, (xiv) an N-acetylamino acid or salt thereof, (xv) an enzyme degradative to a selected membrane component, (ix) an inhibitor of fatty acid synthesis, or (x) an inhibitor of cholesterol synthesis; or (xi) any combination of the membrane penetration enhancing agents recited in (i)-(x);

(h) a second permeabilizing agent that modulates epithelial junction physiology;

(i) a vasodilator agent;

(j) a selective transport-enhancing agent; and

(k) a stabilizing delivery vehicle, carrier, support or complex-forming species with which the biologically active agent is effectively combined, associated, contained, encapsulated or bound resulting in stabilization of the active agent for enhanced mucosal delivery, wherein said one or more mucosal delivery-enhancing agents comprises any one or any combination of two or more of said mucosal delivery-enhancing agents recited in (a)-(k), and wherein the formulation of said biologically active agent with said mucosal delivery-enhancing agents provides for increased bioavailability of the biologically active agent delivered to a mucosal surface of a mammalian subject.

In related aspects of the invention, coordinate administration of the permeabilizing agent and biologically active agent yields a peak concentration (C_{max}) of the biologically active agent in a blood plasma or cerebral spinal fluid (CNS) of the subject that is 25% or greater as compared to a peak concentration of the biologically active agent following intramuscular injection of an equivalent concentration or dose of the active agent to the subject. In additional embodiments, coordinate administration of the permeabilizing agent and biologically active agent yields an area under concentration curve (AUC) of the biologically active agent in a blood plasma or cerebral spinal fluid (CNS) of the subject that is 25% or greater compared to an AUC

of the biologically active agent in blood plasma or CNS following intramuscular injection of an equivalent concentration or dose of the active agent to the subject. In other embodiments, coordinate administration of the permeabilizing agent and biologically active agent yields a time to maximal plasma concentration (t_{max}) of the biologically active agent in a blood plasma or cerebral spinal fluid (CNS) of the subject between 0.2 to 0.5 hours. In still other embodiments, coordinate administration of the permeabilizing agent and biologically active agent yields a peak concentration of the biologically active agent in a central nervous system (CNS) tissue or fluid of the subject that is 10% or greater compared to a peak concentration of the biologically active agent in a blood plasma of the subject.

In yet additional detailed embodiments, the invention provides permeabilizing peptides and peptide analogs and mimetics for enhancing mucosal epithelial paracellular transport. The subject peptides and peptide analogs and mimetics typically work within the compositions and methods of the invention by modulating epithelial junctional structure and/or physiology in a mammalian subject. In certain embodiments, the peptides and peptide analogs and mimetics effectively inhibit homotypic and/or heterotypic binding of an epithelial membrane adhesive protein selected from a junctional adhesion molecule (JAM), occludin, or claudin. In more detailed embodiments, the permeabilizing peptide or peptide analog comprises from about 4-25 contiguous amino acids of a wild-type sequence of an extracellular domain of a mammalian JAM-1, JAM-2, JAM-3, occludin or claudin protein, or an amino acid sequence that exhibits at least 85% amino acid identity with a corresponding reference sequence of about 4-25 contiguous amino acids of a wild-type sequence of an extracellular domain of a mammalian JAM-1, JAM-2, JAM-3, occludin or claudin protein. In exemplary embodiments, the permeabilizing peptide or peptide analog is a human JAM peptide (e.g., human JAM-1) having a wild-type amino acid sequence or exhibiting one or more amino acid mutations in comparison to a corresponding wild-type sequence of the same human JAM protein, wherein the mutation(s) correspond to a structural feature identified in a different human JAM protein or a homologous JAM protein found in a different species.

In more detailed embodiments, the permeabilizing peptide is between about 4-25 amino acids in length, and includes one or more contiguous sequence elements selected from: V R (I, V, A) P (SEQ ID NO: 1); (V, A, I) K L (S, T) C A Y (SEQ ID NO: 2); or E D (T, S) G T Y (T,R) C (M, E) (SEQ ID NO: 3). In one such

embodiment, the peptide will include a conservative sequence motif V R (I, V, A) P (SEQ ID NO: 1), wherein the third position of the motif may be represented by one of the alternative amino acid residues I, V, or A. In another such embodiment, the peptide will include a conservative sequence motif (V, A, I) K L (S, T) C A Y (SEQ ID NO: 2), wherein the first position of the motif may be represented by one of the alternative amino acid residues V, A, or I, and the fourth position of the motif may be represented by one of the alternative amino acid residues S or T. In yet another such embodiment, the peptide will include a conservative sequence motif E D (T, S) G T Y (T,R) C (M, E) (SEQ ID NO: 3), wherein the third position of the motif may be represented by one of the alternative amino acid residues T or S, the seventh position of the motif may be represented by one of the alternative amino acid residues T or R, and the ninth position of the motif may be represented by one of the alternative residues M or E. In exemplary embodiments, the permeabilizing peptide is between about 4-25 amino acids in length and includes one or more contiguous sequence elements selected from wild-type human JAM-1 peptide sequences VRIP (SEQ ID NO: 4), VKLSCAY (SEQ ID NO: 5), and/or EDTGTYTCM (SEQ ID NO: 9).

Permeabilizing peptides for use within the invention include natural or synthetic, therapeutically or prophylactically active, peptides (comprised of two or more covalently linked amino acids), proteins, peptide or protein fragments, peptide or protein analogs, peptide or protein mimetics, and chemically modified derivatives or salts of active peptides or proteins. Thus, as used herein, the term "permeabilizing peptide" will often be intended to embrace all of these active species, i.e., peptides and proteins, peptide and protein fragments, peptide and protein analogs, peptide and protein mimetics, and chemically modified derivatives and salts of active peptides or proteins. Often, the permeabilizing peptides or proteins are muteins that are readily obtainable by partial substitution, addition, or deletion of amino acids within a naturally occurring or native (e.g., wild-type, naturally occurring mutant, or allelic variant) peptide or protein sequence. Additionally, biologically active fragments of native peptides or proteins are included. Such mutant derivatives and fragments substantially retain the desired biological activity of the native peptide or proteins. In the case of peptides or proteins having carbohydrate chains, biologically active

variants marked by alterations in these carbohydrate species are also included within the invention.

The permeabilizing peptides, proteins, analogs and mimetics for use within the methods and compositions of the invention are often formulated in a pharmaceutical composition comprising a mucosal delivery-enhancing or permeabilizing effective amount of the permeabilizing peptide, protein, analog or mimetic that reversibly enhances mucosal epithelial paracellular transport by modulating epithelial junctional structure and/or physiology in a mammalian subject.

In more detailed embodiments of the invention, the permeabilizing agent comprises a peptide of from about 4-25 contiguous amino acids of an extracellular domain of a mammalian JAM-1, JAM-2, or JAM-3 protein. Alternatively, the permeabilizing peptide may comprise from about 6-15 contiguous amino acids of an extracellular domain of a mammalian JAM-1, JAM-2, or JAM-3 protein. In additional embodiments, the permeabilizing peptide comprises from about 4-25 contiguous amino acids of an extracellular domain of a mammalian JAM-1, JAM-2, or JAM-3 protein, or a sequence of amino acids that exhibits at least 85% amino acid identity with a corresponding reference sequence of 4-25 contiguous amino acids of an extracellular domain of a mammalian JAM-1, JAM-2, or JAM-3 protein. In certain embodiments, the amino acid sequence of the permeabilizing peptide exhibits one or more amino acid substitutions, insertions, or deletions compared to a corresponding reference sequence (e.g., a corresponding wild-type sequence) of the mammalian JAM-1, JAM-2, or JAM-3 protein. For example, the permeabilizing peptide may exhibit one or more conservative amino acid substitutions compared to a corresponding reference sequence of a mammalian JAM-1, JAM-2, or JAM-3 protein. Such functional peptide analogs or variants may, for instance, have one or more amino acid mutations in comparison to a corresponding reference sequence of the same human JAM protein (e.g., human JAM-1), wherein the mutation(s) correspond to a divergent amino acid residue or sequence identified in a different human JAM protein (e.g., human JAM-2 or JAM-3) or in a homologous JAM protein found in a different species (e.g. murine, rat, or bovine JAM-1, JAM-2 or JAM-3 protein).

In more detailed embodiments, the methods and compositions of the invention incorporate a permeabilizing peptide that comprises from about 4-25 contiguous amino acids, or from about 6-15 contiguous amino acids, of an extracellular domain of a mammalian JAM-1, JAM-2, or JAM-3 protein. Exemplary permeabilizing

peptides that are demonstrated herein to enhance mucosal permeability for improving mucosal delivery of biologically active agents in mammalian subjects comprise partial sequences of an extracellular domain of a human JAM-1 protein, as exemplified by the peptides VRIP (SEQ ID NO: 4), VKLSCAY (SEQ ID NO: 5), TGITFKSVT (SEQ ID NO: 6), ITAS (SEQ ID NO: 7), SVTR (SEQ ID NO: 8), EDTGTYTCM (SEQ ID NO: 9), and GFSSPRVEW (SEQ ID NO: 10).

Peptides between about 4-25 amino acids in length comprising these and other exemplary sequences may be used directly as permeabilizing agents, or they may be combined in a combinatorial formulation with other mucosal delivery-enhancing agents. In addition, these exemplary peptides may be modified (e.g., by amino- or carboxy-terminal truncation, or addition of flanking amino acid sequences from the corresponding native protein, conjugation to another peptide, carrier, polymer, or biologically active moiety, chemical modification or derivatization, etc.) as described herein. Useful peptides within the invention also include operable sequence variants of the foregoing exemplary peptides, e.g., substitution, deletion, or insertion muteins. For example, by aligning homologous sequences of human and non-human JAM proteins (see the various Tables, and references incorporated herein), the following exemplary candidate JAM-1 peptides for enhancing mucosal permeability are provided: V R (I, V, A) P (SEQ ID NO: 1); (V, A, I) K L (S, T) C A Y (SEQ ID NO: 2); or E D (T, S) G T Y (T,R) C (M, E) (SEQ ID NO: 3), where the residues in parentheses correspond to alternate functional variants predicted, e.g., by alignment of human and non-human JAM proteins to identify sites amenable to mutation and alternate residues present in divergent JAM homologs. This rational design of alternate candidate peptides can include alignments of naturally occurring mutants, allelic variants of a particular JAM, occludin or claudin protein, and by comparisons of different JAM proteins (e.g., JAM-1, JAM-2, JAM-3), as described in further detail below.

According to these rational design methods, exemplary permeabilizing JAM-1 peptide candidates include, but are not limited to VRIP (SEQ ID NO: 4), VRVP (SEQ ID NO: 11), VRAP (SEQ ID NO: 12), PVRIPE (SEQ ID NO: 13), PEVRIPEN (SEQ ID NO: 14), EPEVRIPENN (SEQ ID NO: 15), SEPEVRIPENNP (SEQ ID NO: 16), SSEPEVRIPENNPV (SEQ ID NO: 17), HSSEPEVRIPENNPVK (SEQ ID NO: 18), VHSSEPEVRIPENNPVKL (SEQ ID NO: 19), and TVHSSEPEVRIPENNPVKLS (SEQ ID NO: 20). Further exemplary permeabilizing JAM-1 peptide candidates

include, but are not limited to VKLSCAY (SEQ ID NO: 5), AKLSCAY (SEQ ID NO: 21), IKLSCAY (SEQ ID NO: 22), VKLTCAY (SEQ ID NO: 23), AKLTCAY (SEQ ID NO: 24), and IKLTCAY (SEQ ID NO: 25). Yet additional exemplary permeabilizing JAM-1 peptide candidates include, but are not limited to EDTGTYTCM (SEQ ID NO: 9), EDTGTYTCE (SEQ ID NO: 25), EDTGTYRCM (SEQ ID NO: 26), EDTGTYRCE (SEQ ID NO: 27), EDSGTYTCM (SEQ ID NO: 28), EDSGTYTCE (SEQ ID NO: 29), EDSGTYRCM (SEQ ID NO: 30), EDSGTYRCE (SEQ ID NO: 31).

Conservative amino acid substitutions within exemplary permeabilizing JAM-1 peptides may be determined by comparison of conserved sequences within the extracellular domain of JAM-1 from human (AF111713; Ozaki, et al., J. Immunol. 163:553-557, 1999,); Williams et al. (Mol. Immunol. 36:1175-1188, 1999; Liu et al., J. Cell. Sci. 113:2363-2374, 2000; Sobocka et al., Blood 95:2600-2609, 2000,), murine (U89915; Martin-Padura, et al., J. Cell Biol. 142:117-127, 1998; Malergue, et al., Mol Immunol. 35:1111-1119, 1998,), bovine (AF111714; Ozaki, et al., J. Immunol. 163:553-557, 1999,) and rat (AF276998, Direct submission to ENTREZ/GenBank database, National Center for Biotechnology Information,). (See Table 1, below).

As further summarized in Table 1, nomenclature for human JAM-1, JAM-2, and JAM-3 is clarified in Dejana, et al., Thromb. Haemost. 86: 308-315, 2001. The human JAM-2 sequence is found at AJ344431 (Aurrand-Lions, Direct submission to ENTREZ/GenBank database, National Center for Biotechnology Information,), AF356518 (Pia Arrate, et al., J. Biol. Chem. 276:45826-45832, 2001,), and AX036049 to AX036065 (Aurrand-Lions, WO 00/53749,). Mouse JAM-2 is found at AJ300304 (Aurrand-Lions, et al., J. Biol. Chem. 276:2733-2741, 2001). Human JAM-3 is found at AF255910 (Palmeri, et al., J. Biol. Chem. 275:19139-19145, 2000), and AY016009 (Cunningham, et al. J. Biol. Chem. 275:34750-34756, 2000). Mouse JAM-3 is found at AF255911 (Palmeri, et al., J. Biol. Chem. 275:19139-19145, 2000).

Table 1: Reference Survey of JAM Proteins

Name	Species	Genbank #	References
JAM-1 (JAM, F11)	Human	AF111713	Ozaki, et al., 1999; Williams et al., 1999; Liu, et al., 2000; Sobocka, et al., 2000.
	Mouse	U89915	Martin-Padura, et al., 1998; Malergue, et al., 1998.
	Bovine	AF111714	Ozaki, et al., 1999.
	Rat	AF276998	Submitted 6/12/00.
JAM-2 (JAM-3)			
	Human	AJ344431---------→ AF356518---------→ AX036049 to AX036065--------→	Submitted 8/28/01; (Aurrand-Lions) Pia Arrate, et al., 2001. (= JAM-3) WO 00/53749 (Aurrand-Lions)
	Mouse	AJ300304	Aurrand-Lions, et al., <u>J. Biol. Chem</u>. 276: 2733-2741 (2001)
JAM-3 (VE-JAM, JAM-2)			
	Human	AF255910--------→ AY016009--------→	Palmeri, et al., 2000 (=VE-JAM) Cunningham, et al. 2000 (= JAM-2)
	mouse	AF255911	Palmeri, et al., 2000

Within additional aspects of the invention, pharmaceutical compositions and methods are provided that employ a permeabilizing peptide comprising from about 4-25 contiguous amino acids of an extracellular domain of a mammalian occludin protein. In alternate embodiments, the permeabilizing peptide comprises from about 6-15 contiguous amino acids of an extracellular domain of a mammalian occludin protein. In certain aspects, the permeabilizing peptide comprises from about 4-25 contiguous amino acids of an extracellular domain of a mammalian occludin protein or comprises an amino acid sequence that exhibits at least 85% amino acid identity with a corresponding reference sequence of 4-25 contiguous amino acids of an

extracellular domain of a mammalian occludin protein. In exemplary embodiments, the permeabilizing peptide exhibits one or more amino acid substitutions, insertions, or deletions compared to a corresponding reference sequence of the mammalian occludin protein. Often, such peptide analogs will exhibit one or more conservative amino acid substitutions compared to the corresponding reference sequence of the mammalian occludin protein. In related embodiments, the permeabilizing peptide is a human occludin peptide and the amino acid sequence of the permeabilizing peptide exhibits one or more amino acid mutations in comparison to a corresponding reference sequence (e.g., wild-type sequence) of the same human occludin protein, wherein the mutation(s) correspond to a structural feature (e.g., a divergent, aligned residue or sequence of residues) identified in a different human occludin protein or a homologous occludin protein found in a different species.

Exemplary occludin peptides that demonstrate efficacy to enhance mucosal permeability to faclitate delivery of a biologically active agent in a mammalian subject comprise from about 4-25 contiguous amino acids of an extracellular domain of human occludin protein, as exemplified by the operable peptides: AATGLYVDQ (SEQ ID NO: 32), GVNPTAQSS (SEQ ID NO: 33), GSLYGSQIY (SEQ ID NO: 34), ALCNQFYTP (SEQ ID NO: 35, and YLYHYCVVD (SEQ ID NO: 36).

Taking the first of these operable peptides as a reference sequence, additional peptides that comprise this peptide and may optionally include other residues from the native occludin extracellular domain sequence will include, for example: AATGLYVDQ (SEQ ID NO: 32), PAATGLYVDQY (SEQ ID NO: 37), TPAATGLYVDQYL (SEQ ID NO: 38), YTPAATGLYVDQYLY (SEQ ID NO: 39), FYTPAATGLYVDQYLYH (SEQ ID NO: 40), QFYTPAATGLYVDQYLYHY (SEQ ID NO: 41), YLYHYCVVD (SEQ ID NO: 42), QYLYHYCVVDP (SEQ ID NO: 43), DQYLYHYCVVDPQ (SEQ ID NO: 44), VDQYLYHYCVVDPQE (SEQ ID NO: 45), YVDQYLYHYCVVDPQEA (SEQ ID NO: 46), LYVDQYLYHYCVVDPQEAI (SEQ ID NO: 47), AATGLYVDQ (SEQ ID NO: 48), ATGLYVD (SEQ ID NO: 49), TGLYVD (SEQ ID NO: 50), TGLYV (SEQ ID NO: 51) and GLYV (SEQ ID NO: 52).

Within other aspects of the invention, pharmaceutical compositions and methods are provided that employ a permeabilizing peptide comprising from about 4-25 contiguous amino acids of an extracellular domain of a mammalian claudin protein. In alternate embodiments, the permeabilizing peptide comprises from about

6-15 contiguous amino acids of an extracellular domain of a mammalian claudin protein. In certain aspects, the permeabilizing peptide comprises from about 4-25 contiguous amino acids of an extracellular domain of a mammalian claudin protein or comprises an amino acid sequence that exhibits at least 85% amino acid identity with a corresponding reference sequence of 4-25 contiguous amino acids of an extracellular domain of a mammalian claudin protein. In exemplary embodiments, the permeabilizing peptide exhibits one or more amino acid substitutions, insertions, or deletions compared to a corresponding reference sequence of the mammalian claudin protein. Often, such peptide analogs will exhibit one or more conservative amino acid substitutions compared to the corresponding reference sequence of the mammalian claudin protein. In related embodiments, the permeabilizing peptide is a human claudin peptide and the amino acid sequence of the permeabilizing peptide exhibits one or more amino acid mutations in comparison to a corresponding wild-type sequence of the same human claudin protein, wherein the mutation(s) correspond to a structural feature (e.g., a divergent, aligned residue or sequence of residues) identified in a different human claudin protein or a homologous claudin protein found in a different species.

In more detailed aspects of the invention, exemplary permeabilizing peptides for enhancing mucosal delivery of a biologically active agent in a mammalian subject comprise from about 4-25 amino acids of an extracellular domain of a human claudin protein include biologically active peptide or protein analogs of the extracellular domain of a human claudin protein. Exemplary permeabilizing peptides comprise amino acids of an extracellular domain of human claudin-1, claudin-2, claudin-3, claudin-4, claudin-5, claudin-6, claudin-7, claudin-8, claudin-9, claudin-10, claudin-11, claudin-12, claudin-13, claudin-14, claudin-15, claudin-16, claudin-17, claudin-18, claudin-19, or claudin-20. Exemplary permeabilizing peptides within this aspect of the invention that demonstrate efficacy to enhance mucosal permeability to facilitate delivery of a biologically active agent in a mammalian subject include, but are not limited to: GILRDFYSPL (SEQ ID NO: 53), NTIIRDFYNP (SEQ ID NO: 54), DIYSTLLGLP (SEQ ID NO: 55), GFSLGLWMEC (SEQ ID NO: 56), YAGDNIVTAQ (SEQ ID NO: 57), MTPVNARYEF (SEQ ID NO: 58), VASGQKREMG (SEQ ID NO: 59), VPDSMKFEIG (SEQ ID NO: 60), NIIQDFYNPL (SEQ ID NO: 61), VPVSQKYELG (SEQ ID NO: 62), and VVPEAQKREM (SEQ ID NO: 63).

Taking the first of these operable peptides as a reference sequence, additional peptides that comprise this peptide and may optionally include other residues from the native occludin extracellular domain sequence will include, for example: GILRDFYSPL (SEQ ID NO: 53), HGILRDFYSPLV (SEQ ID NO: 64), LHGILRDFYSPLVP (SEQ ID NO: 65), NLHGILRDFYSPLVPD (SEQ ID NO: 66), GILRDFYSPLVPDS (SEQ ID NO: 67), GILRDFYSPLVPDSM (SEQ ID NO: 68), GILRDFYSPLVPDSMK (SEQ ID NO: 69), GILRDFYSPLVPDSMKF (SEQ ID NO: 70), GILRDFYSPLVPDSMKFE (SEQ ID NO: 71), GILRDFYSPL (SEQ ID NO: 53), ILRDFYSP (SEQ ID NO: 72), LRDFYS (SEQ ID NO: 73), RDFYS (SEQ ID NO: 74), and RDFY (SEQ ID NO: 75). Additional exemplary permeabilizing peptides comprising amino acids of an extracellular domain of human claudin protein include, but are not limited to: NIIQDFYNPL (SEQ ID NO: 61), HNIIQDFYNPLV (SEQ ID NO: 76), AHNIIQDFYNPLVA (SEQ ID NO: 77), TAHNIIQDFYNPLVAS (SEQ ID NO: 78), WTAHNIIQDFYNPLVASG (SEQ ID NO: 79), SWTAHNIIQDFYNPLVASGQ (SEQ ID NO: 80), and VSWTAHNIIQDFYNPLVASGQK (SEQ ID NO: 81). Yet additional exemplary permeabilizing peptides comprising amino acids of an extracellular domain of human claudin protein include, but are not limited to: VVPEAQKREM (SEQ ID NO: 63), PVVPEAQKREMG (SEQ ID NO: 82), NPVVPEAQKREMGA (SEQ ID NO: 83), YNPVVPEAQKREMGAG (SEQ ID NO: 84), FYNPVVPEAQKREMGAGL (SEQ ID NO: 85), DFYNPVVPEAQKREMGAGLY (SEQ ID NO: 86), RDFYNPVVPEAQKREMGAGLYV (SEQ ID NO: 87), and IRDFYNPVVPEAQKREMGAGLYVG (SEQ ID NO: 88).

Exemplary permeabilizing peptides that enhance mucosal delivery of a biologically active agent in a mammalian subject (e.g., a human JAM-1, JAM-2, JAM-3, human claudin-1 through claudin-20, or human occludin proteins will typically exhibit a significant permeabilizing effect on mucosal epithelia. For example, this effect may be demonstrated as an effect on trans epithelial electrical resistance (TER) in a suitable *in vitro* epithelial permeability model, such as the EpiAirway™ Cell Membrane model system widely accepted in the art as a model for epithelial barrier functionality. Exemplary permeabilizing peptides demonstrate a decrease in TER in an EpiAirway™ Cell Membrane compared to valid controls (e.g., in the absence of permeabilizing peptides). Exemplary permeabilizing peptides

corresponding to a human JAM-1, JAM-2, JAM-3, non-human JAM, claudin or occludin extracellular domain sequence, or an active analog or mimetic thereof, will typically cause a measurable reduction of TER in these model systems, often to at least 85% or less compared to control TER values. Often, permeabilizing peptides of the invention will yield a reduction in TER value to at least 75% or less compared to control values. In certain embodiments, the permeabilizing peptides will yield a reduction in TER value to least 60% or less compared to control values. In still more effective embodiments, the human JAM-1, JAM-2, JAM-3, non-human JAM, claudin or occludin peptides, or active analogs and mimetics thereof, will yield a reduction in TER of at least 50% or less compared to control values.

Human JAM-1 is a polypeptide of 299 amino acids having a predicted extracellular domain (underlined in Figure 1) from amino acids 28 to 235. Exemplary candidate permeabilizing peptides, having a length of between about 4-25 amino acids and comprising a portion of the JAM-1 extracellular domain, are shown in Tables 2-5, below. Table 2 presents four panels of scanning peptides from the extracellular domain of human JAM-1 from which candidate permeabilizing peptides will be screened and validated for use within the invention. By these methods, the above-noted exemplary permeabilizing peptides of human JAM-1 (VRIP (SEQ ID NO: 4), VKLSCAY (SEQ ID NO: 5), TGITFKSVT (SEQ ID NO: 6), ITAS (SEQ ID NO: 7), SVTR (SEQ ID NO: 8), EDTGTYTCM (SEQ ID NO: 9), and GFSSPRVEW (SEQ ID NO: 10)) were identified. Following the description and teachings herein, additional permeabilizing peptides (i.e., peptides that operate to measurably increase mucosal epithelial permeability, e.g., by reducing TER and/or increasing rates of transport of macromolecules across mucosal epithelial cell layers in culture, or across mucosal tissues and/or into selected tissues or physiological compartments of a mammalian subject *in vivo*) will be readily identified and incorporated within the methods and compositions of the invention.

Table 2. Candidate Permeabilizing Peptides of Human JAM-1 (Scanning peptides)

	SEQ ID NO:		SEQ ID NO:
PANEL 1		**PANEL 2**	
SVTVHSSEPE	89	SVTVHSSE	110
VRIPENNPVK	90	PEVRIPEN	111
LSCAYSGFSS	91	NPVKLSCA	112
PRVEWKFDQG	92	YSGFSSPR	113
DTTRLVCYNN	93	VEWKFDQG	114
KITASYEDRV	94	DTTRLVCY	115
TFLPTGITFK	95	NNKITASY	116
SVTREDTGTY	96	EDRVTFLP	117
TCMVSEEGGN	97	TGITFKSV	118
SYGEVKVKLI	98	TREDTGTY	119
VLVPPSKPTV	99	TCMVSEEG	120
NIPSSATIGN	100	GNSYGEVK	121
RAVLTCSEQD	101	VKLIVLVP	122
GSPPSEYTWF	102	PSKPTVNI	123
KDGIVMPTNP	103	PSSATIGN	124
KSTRAFSNSS	104	RAVLTCSE	125
YVLNPTTGEL	105	QDGSPPSE	126
VFDPLSASDT	106	YTWFKDGI	127
GEYSCEARNG	107	VMPTNPKS	128
YGTPMTSNAV	108	TRAFSNSS	129
RMEAVERNVG	109	YVLNPTTG	130
		ELVFDPLS	131
PANEL 3		ASDTGEYS	132
		CEARNGYG	133
SVTVH	137	TPMTSNAV	134
SSEPEVRIPE	138	RMEAVERN	135
NNPVKLSCAY	139	VGVI	136
SGFSSPRVEW	140		
KFDQGDTTRL	141	**PANEL 4**	
VCYNNKITAS	142		
YEDRVTFLPT	143	SVTV	159
GITFKSVTRE	144	HSSEPEVR	160
DTGTYTCMVS	145	IPENNPVK	161
EEGGNSYGEV	146	LSCAYSGF	162
KVKLIVLVPP	147	SSPRVEWK	163
SKPTVNIPSS	148	FDQGDTTR	164
ATIGNRAVLT	149	LVCYNNKI	165
CSEQDGSPPS	150	TASYEDRV	166
EYTWFKDGIV	151	TFLPTGIT	167
MPTNPKSTRA	152	FKSVTRED	168
FSNSSYVLNP	153	TGTYTCMV	169
TTGELVFDPL	154	SEEGGNSY	170
SASDTGEYSC	155	GEVKVKLI	171
EARNGYGTPM	156	VLVPPSKP	172
TSNAVRMEAV	157	TVNIPSSA	173
ERNVGVI	158	TIGNRAVL	174
		TCSEQDGS	175
		PPSEYTWF	176
		KDGIVMPT	177
		NPKSTRAF	178
		SNSSYVLN	179
		PTTGELVF	180
		DPLSASDT	181
		GEYSCEAR	182
		NGYGTPMT	183
		SNAVRMEA	184
		VERNVGVI	185

In addition to the above-noted exemplary permeabilizing peptides of human JAM-1 (VRIP (SEQ ID NO: 4), VKLSCAY (SEQ ID NO: 5), TGITFKSVT (SEQ ID NO: 6), ITAS (SEQ ID NO: 7), SVTR (SEQ ID NO: 8), EDTGTYTCM (SEQ ID NO: 9), and GFSSPRVEW (SEQ ID NO: 10)), various analogs and mimetics of these peptides as described herein are provided. Tables 3-5 set forth three exemplary panels of peptide analogs based on exemplary human JAM-1 peptides that have been shown to be particularly active within the methods and compositions of the invention. These panels of peptide analog candidates for increasing mucosal permeability include sequence variants of the foregoing exemplary peptides predicted to be operable, for example, by aligning homologous sequences of human and non-human JAM proteins. From these alignments, conservative peptide motifs are determined, as exemplified by the conservative motifs: V R (I, V, A) P (SEQ ID NO:1); (V, A, I) K L (S, T) C A Y (SEQ ID NO: 2); or E D (T, S) G T Y (T,R) C (M, E) (SEQ ID NO: 3). As noted above, these motifs share strictly conserved residues, and divergent residues (shown in parentheses) that are expected to be interchangeable to yield a functional JAM-1 peptide analog.

In accordance with these rational design methods, certain embodiments of the invention will include a JAM permeabilizing peptide that comprises a conservative sequence motif V R (I, V, A) P (SEQ ID NO: 1), wherein the third position of the motif may be represented by one of the alternative amino acid residues I, V, or A. In another such embodiment, the peptide will include a conservative sequence motif (V, A, I) K L (S, T) C A Y (SEQ ID NO: 2), wherein the first position of the motif may be represented by one of the alternative amino acid residues V, A, or I, and the fourth position of the motif may be represented by one of the alternative amino acid residues S or T. In yet another such embodiment, the peptide will include a conservative sequence motif E D (T, S) G T Y (T,R) C (M, E) (SEQ ID NO: 3), wherein the third position of the motif may be represented by one of the alternative amino acid residues T or S, the seventh position of the motif may be represented by one of the alternative amino acid residues T or R, and the ninth position of the motif may be represented by one of the alternative residues M or E. In accordance with these teachings, Tables 3-5 set forth three such exemplary panels of peptide analogs. Included within these panels of peptide analogs are peptides that include amino- or carboxy-terminal extensions in comparison to the documented reference JAM-1 peptide, which

extensions will typically correspond to corresponding flanking sequences of the native JAM-1 protein as shown.

Table 3. Exemplary Permeabilizing Peptides of Human JAM-1

	SEQ ID NO:		SEQ ID NO:
VRIP		V R (V) P	212
V R (I, V, A) P	1	PV R (V) PE	213
V R (I) P	4	PEV R (V) PEN	214
PV R (I) PE	186	EPEV R (V) PENN	215
PEV R (I) PEN	187	SEPEV R (V) PENNP	216
EPEV R (I) PENN	188	SSEPEV R (V) PENNPV	217
SEPEV R (I) PENNP	189	HSSEPEV R (V) PENNPVK	218
SSEPEV R (I) PENNPV	190	VHSSEPEV R (V) PENNPVKL	219
HSSEPEV R (I) PENNPVK	191	TVHSSEPEV R (V)	220
VHSSEPEV R (I)	192	PENNPVKLS	221
PENNPVKL	193	V R (V) PE	222
TVHSSEPEV R (I)	194	V R (V) PEN	223
PENNPVKLS	195	V R (V) PENN	224
V R (I) PE	196	V R (V) PENNP	225
V R (I) PEN	197	V R (V) PENNPV	226
V R (I) PENN	198	V R (V) PENNPVK	227
V R (I) PENNP	199	V R (V) PENNPVKL	228
V R (I) PENNPV	200	V R (V) PENNPVKLS	229
V R (I) PENNPVK	201	EV R (V) P	230
V R (I) PENNPVKL	202	PEV R (V) P	231
V R (I) PENNPVKLS	203	EPEV R (V) P	232
EV R (I) P	204	SEPEV R (V) P	233
PEV R (I) P	205	SSEPEV R (V) P	234
EPEV R (I) P	206	HSSEPEV R (V) P	235
SEPEV R (I) P	207	VHSSEPEV R (V) P	236
SSEPEV R (I) P	208	TVHSSEPEV R (V) P	237
HSSEPEV R (I) P	209	V R (A) P	238
VHSSEPEV R (I) P	210	PV R (A) PE	239
TVHSSEPEV R (I) P	211	PEV R (A) PEN	240
		EPEV R (A) PENN	241
		SEPEV R (A) PENNP	242
		SSEPEV R (A) PENNPV	243
		HSSEPEV R (A) PENNPVK	244
		VHSSEPEV R (A) PENNPVKL	245
		TVHSSEPEV R (A)	246
		PENNPVKLS	247
		V R (A) PE	248
		V R (A) PEN	249
		V R (A) PENN	250
		V R (A) PENNP	251
		V R (A) PENNPV	252
		V R (A) PENNPVK	253
		V R (A) PENNPVKL	254
		V R (A) PENNPVKLS	255
		EV R (A) P	256
		PEV R (A) P	257
		EPEV R (A) P	258
		SEPEV R (A) P	259
		SSEPEV R (A) P	260
		HSSEPEV R (A) P	261
		VHSSEPEV R (A) P	262
		TVHSSEPEV R (A) P	263

Table 4. Exemplary Permeabilizing Peptides of Human JAM-1

	SEQ ID NO:		SEQ ID NO:
VKLSCAY		(A) K L (S) C A Y	308
(V, A, I) K L (S, T) C A Y	2	(A) K L (S) C A Y	309
(V) K L (S) C A Y	5	P (A) K L (S) C A Y S	310
P (V) K L (S) C A Y S	264	NP (A) K L (S) C A Y SG	311
NP (V) K L (S) C A Y SG	265	NNP (A) K L (S) C A Y SGF	312
NNP (V) K L (S) C A Y SGF	266	ENNP (A) K L (S) C A Y SGFS	313
ENNP (V) K L (S) C A Y SGFS	267	PENNP (A) K L (S) C A Y SGFSS	314
PENNP (V) K L (S) C A Y SGFSS	268	IPENNP (A) K L (S) C A Y SGFSSP	315
IPENNP (V) K L (S) C A Y SGFSSP	269	RIPENNP (A) K L (S) C A Y SGFSSPR	316
RIPENNP (V) K L (S) C A Y SGFSSPR	270	P (A) K L (S) C A Y	317
P (V) K L (S) C A Y	271	NP (A) K L (S) C A Y	318
NP (V) K L (S) C A Y	272	NNP (A) K L (S) C A Y	319
NNP (V) K L (S) C A Y	273	ENNP (A) K L (S) C A Y	320
ENNP (V) K L (S) C A Y	274	PENNP (A) K L (S) C A Y	321
PENNP (V) K L (S) C A Y	275	IPENNP (A) K L (S) C A Y	322
IPENNP (V) K L (S) C A Y	276	RIPENNP (A) K L (S) C A Y	323
RIPENNP (V) K L (S) C A Y	277	(A) K L (S) C A Y S	324
(V) K L (S) C A Y S	278	(A) K L (S) C A Y SG	325
(V) K L (S) C A Y SG	279	(A) K L (S) C A Y SGF	326
(V) K L (S) C A Y SGF	280	(A) K L (S) C A Y SGFS	327
(V) K L (S) C A Y SGFS	281	(A) K L (S) C A Y SGFSS	328
(V) K L (S) C A Y SGFSS	282	(A) K L (S) C A Y SGFSSP	329
(V) K L (S) C A Y SGFSSP	283	(A) K L (S) C A Y SGFSSPR	330
(V) K L (S) C A Y SGFSSPR	284	(A) K L (T) C A Y	331
(V) K L (T) C A Y	285	(A) K L (T) C A Y	332
(V) K L (T) C A Y	286	P (A) K L (T) C A Y S	333
P (V) K L (T) C A Y S	287	NP (A) K L (T) C A Y SG	334
NP (V) K L (T) C A Y SG	288	NNP (A) K L (T) C A Y SGF	335
NNP (V) K L (T) C A Y SGF	289	ENNP (A) K L (T) C A Y SGFS	336
ENNP (V) K L (T) C A Y SGFS	290	PENNP (A) K L (T) C A Y SGFSS	337
PENNP (V) K L (T) C A Y SGFSS	291	IPENNP (A) K L (T) C A Y SGFSSP	338
IPENNP (V) K L (T) C A Y SGFSSP	292	RIPENNP (A) K L (T) C A Y SGFSSPR	339
RIPENNP (V) K L (T) C A Y SGFSSPR	293	P (A) K L (T) C A Y	340
P (V) K L (T) C A Y	294	NP (A) K L (T) C A Y	341
NP (V) K L (T) C A Y	295	NNP (A) K L (T) C A Y	342
NNP (V) K L (T) C A Y	296	ENNP (A) K L (T) C A Y	343
ENNP (V) K L (T) C A Y	297	PENNP (A) K L (T) C A Y	344
PENNP (V) K L (T) C A Y	298	IPENNP (A) K L (T) C A Y	345
IPENNP (V) K L (T) C A Y	299	RIPENNP (A) K L (T) C A Y	346
RIPENNP (V) K L (T) C A Y	300	(A) K L (T) C A Y S	347
(V) K L (T) C A Y S	301	(A) K L (T) C A Y SG	348
(V) K L (T) C A Y SG	302	(A) K L (T) C A Y SGF	349
(V) K L (T) C A Y SGF	303	(A) K L (T) C A Y SGFS	350
(V) K L (T) C A Y SGFS	304	(A) K L (T) C A Y SGFSS	351
(V) K L (T) C A Y SGFSS	305	(A) K L (T) C A Y SGFSSP	352
(V) K L (T) C A Y SGFSSP	306	(A) K L (T) C A Y SGFSSPR	353
(V) K L (T) C A Y SGFSSPR	307		

Table 4. Exemplary Permeabilizing Peptides of Human JAM-1 (Continued)

	SEQ ID NO:		SEQ ID NO:
(I) K L (S) C A Y	354	(I) K L (T) C A Y	376
(I) K L (S) C A Y	354	(I) K L (T) C A Y	376
P (I) K L (S) C A Y S	355	P (I) K L (T) C A Y S	377
NP (I) K L (S) C A Y SG	356	NP (I) K L (T) C A Y SG	378
NNP (I) K L (S) C A Y SGF	357	NNP (I) K L (T) C A Y SGF	379
ENNP (I) K L (S) C A Y SGFS	358	ENNP (I) K L (T) C A Y SGFS	380
PENNP (I) K L (S) C A Y SGFSS	359	PENNP (I) K L (T) C A Y SGFSS	381
IPENNP (I) K L (S) C A Y SGFSSP	360	IPENNP (I) K L (T) C A Y SGFSSP	382
RIPENNP (I) K L (S) C A Y SGFSSPR	361	RIPENNP (I) K L (T) C A Y SGFSSPR	383
P (I) K L (S) C A Y	362	P (I) K L (T) C A Y	384
NP (I) K L (S) C A Y	363	NP (I) K L (T) C A Y	385
NNP (I) K L (S) C A Y	364	NNP (I) K L (T) C A Y	386
ENNP (I) K L (S) C A Y	365	ENNP (I) K L (T) C A Y	387
PENNP (I) K L (S) C A Y	366	PENNP (I) K L (T) C A Y	388
IPENNP (I) K L (S) C A Y	367	IPENNP (I) K L (T) C A Y	389
RIPENNP (I) K L (S) C A Y	368	RIPENNP (I) K L (T) C A Y	390
(I) K L (S) C A Y S	369	(I) K L (T) C A Y S	391
(I) K L (S) C A Y SG	370	(I) K L (T) C A Y SG	392
(I) K L (S) C A Y SGF	371	(I) K L (T) C A Y SGF	393
(I) K L (S) C A Y SGFS	372	(I) K L (T) C A Y SGFS	394
(I) K L (S) C A Y SGFSS	373	(I) K L (T) C A Y SGFSS	395
(I) K L (S) C A Y SGFSSP	374	(I) K L (T) C A Y SGFSSP	396
(I) K L (S) C A Y SGFSSPR	375	(I) K L (T) C A Y SGFSSPR	397

Table 5. Exemplary Permeabilizing Peptides of Human JAM-1

	SEQ ID NO:		SEQ ID NO:
EDTGTYTCM	9	E D (T) G T Y (R) C (E)	445
E D (T, S) G T Y (T,R) C (M, E)	3	R E D (T) G T Y (R) C (E) V	446
E D (T) G T Y (T) C (M)	9	TR E D (T) G T Y (R) C (E) VS	447
R E D (T) G T Y (T) C (M) V	398	VTR E D (T) G T Y (R) C (E) VSE	448
TR E D (T) G T Y (T) C (M) VS	399	SVTR E D (T) G T Y (R) C (E) VSEE	449
VTR E D (T) G T Y (T) C (M) VSE	400	KSVTR E D (T) G T Y (R) C (E) VSEEG	450
SVTR E D (T) G T Y (T) C (M) VSEE	401	R E D (T) G T Y (R) C (E)	451
KSVTR E D (T) G T Y (T) C (M) VSEEG	402	TR E D (T) G T Y (R) C (E)	452
R E D (T) G T Y (T) C (M)	403	VTR E D (T) G T Y (R) C (E)	453
TR E D (T) G T Y (T) C (M)	404	SVTR E D (T) G T Y (R) C (E)	454
VTR E D (T) G T Y (T) C (M)	405	KSVTR E D (T) G T Y (R) C (E)	455
SVTR E D (T) G T Y (T) C (M)	406	E D (T) G T Y (R) C (E) V	456
KSVTR E D (T) G T Y (T) C (M)	407	E D (T) G T Y (R) C (E) VS	457
E D (T) G T Y (T) C (M) V	408	E D (T) G T Y (R) C (E) VSE	458
E D (T) G T Y (T) C (M) VS	409	E D (T) G T Y (R) C (E) VSEE	459
E D (T) G T Y (T) C (M) VSE	410	E D (T) G T Y (R) C (E) VSEEG	460
E D (T) G T Y (T) C (M) VSEE	411	E D (S) G T Y (T) C (M)	461
E D (T) G T Y (T) C (M) VSEEG	412	R E D (S) G T Y (T) C (M) V	462
E D (T) G T Y (T) C (E)	413	TR E D (S) G T Y (T) C (M) VS	463
R E D (T) G T Y (T) C (E) V	414	VTR E D (S) G T Y (T) C (M) VSE	464
TR E D (T) G T Y (T) C (E) VS	415	SVTR E D (S) G T Y (T) C (M) VSEE	465
VTR E D (T) G T Y (T) C (E) VSE	416	KSVTR E D (S) G T Y (T) C (M) VSEEG	466
SVTR E D (T) G T Y (T) C (E) VSEE	417	R E D (S) G T Y (T) C (M)	467
KSVTR E D (T) G T Y (T) C (E) VSEEG	418	TR E D (S) G T Y (T) C (M)	468
R E D (T) G T Y (T) C (E)	419	VTR E D (S) G T Y (T) C (M)	469
TR E D (T) G T Y (T) C (E)	420	SVTR E D (S) G T Y (T) C (M)	470
VTR E D (T) G T Y (T) C (E)	421	KSVTR E D (S) G T Y (T) C (M)	471
SVTR E D (T) G T Y (T) C (E)	422	E D (S) G T Y (T) C (M) V	472
KSVTR E D (T) G T Y (T) C (E)	423	E D (S) G T Y (T) C (M) VS	473
E D (T) G T Y (T) C (E) V	424	E D (S) G T Y (T) C (M) VSE	474
E D (T) G T Y (T) C (E) VS	425	E D (S) G T Y (T) C (M) VSEE	475
E D (T) G T Y (T) C (E) VSE	426	E D (S) G T Y (T) C (M) VSEEG	476
E D (T) G T Y (T) C (E) VSEE	427	E D (S) G T Y (T) C (E)	477
E D (T) G T Y (T) C (E) VSEEG	428	R E D (S) G T Y (T) C (E) V	478
E D (T) G T Y (R) C (M)	429	TR E D (S) G T Y (T) C (E) VS	479
R E D (T) G T Y (R) C (M) V	430	VTR E D (S) G T Y (T) C (E) VSE	480
TR E D (T) G T Y (R) C (M) VS	431	SVTR E D (S) G T Y (T) C (E) VSEE	481
VTR E D (T) G T Y (R) C (M) VSE	432	KSVTR E D (S) G T Y (T) C (E) VSEEG	482
SVTR E D (T) G T Y (R) C (M) VSEE	433	R E D (S) G T Y (T) C (E)	483
KSVTR E D (T) G T Y (R) C (M) VSEEG	434	TR E D (S) G T Y (T) C (E)	484
R E D (T) G T Y (R) C (M)	435	VTR E D (S) G T Y (T) C (E)	485
TR E D (T) G T Y (R) C (M)	436	SVTR E D (S) G T Y (T) C (E)	486
VTR E D (T) G T Y (R) C (M)	437	KSVTR E D (S) G T Y (T) C (E)	487
SVTR E D (T) G T Y (R) C (M)	438	E D (S) G T Y (T) C (E) V	488
KSVTR E D (T) G T Y (R) C (M)	439	E D (S) G T Y (T) C (E) VS	489
E D (T) G T Y (R) C (M) V	440	E D (S) G T Y (T) C (E) VSE	490
E D (T) G T Y (R) C (M) VS	441	E D (S) G T Y (T) C (E) VSEE	491
E D (T) G T Y (R) C (M) VSE	442	E D (S) G T Y (T) C (E) VSEEG	492
E D (T) G T Y (R) C (M) VSEE	443		
E D (T) G T Y (R) C (M) VSEEG	444		

Table 5. Exemplary Permeabilizing Peptides of Human JAM-1 (Continued)

	SEQ ID NO:		SEQ ID NO:
E D (S) G T Y (R) C (M)	493	E D (S) G T Y (R) C (E)	509
R E D (S) G T Y (R) C (M) V	494	R E D (S) G T Y (R) C (E) V	510
TR E D (S) G T Y (R) C (M) VS	495	TR E D (S) G T Y (R) C (E) VS	511
VTR E D (S) G T Y (R) C (M) VSE	496	VTR E D (S) G T Y (R) C (E) VSE	512
SVTR E D (S) G T Y (R) C (M) VSEE	497	SVTR E D (S) G T Y (R) C (E) VSEE	513
KSVTR E D (S) G T Y (R) C (M) VSEEG	498	KSVTR E D (S) G T Y (R) C (E) VSEEG	514
R E D (S) G T Y (R) C (M)	499	R E D (S) G T Y (R) C (E)	515
TR E D (S) G T Y (R) C (M)	500	TR E D (S) G T Y (R) C (E)	516
VTR E D (S) G T Y (R) C (M)	501	VTR E D (S) G T Y (R) C (E)	517
SVTR E D (S) G T Y (R) C (M)	502	SVTR E D (S) G T Y (R) C (E)	518
KSVTR E D (S) G T Y (R) C (M)	503	KSVTR E D (S) G T Y (R) C (E)	519
E D (S) G T Y (R) C (M) V	504	E D (S) G T Y (R) C (E) V	520
E D (S) G T Y (R) C (M) VS	505	E D (S) G T Y (R) C (E) VS	521
E D (S) G T Y (R) C (M) VSE	506	E D (S) G T Y (R) C (E) VSE	522
E D (S) G T Y (R) C (M) VSEE	507	E D (S) G T Y (R) C (E) VSEE	523
E D (S) G T Y (R) C (M) VSEEG	508	E D (S) G T Y (R) C (E) VSEEG	524

Human JAM-2 is a polypeptide of 310 amino acids having a predicted extracellular domain from amino acids 31 to 241. The full-length sequence of JAM-2 is provided and the extracellular domain is underlined in Figure 2. Table 6 presents four panels of scanning peptides from the extracellular domain of human JAM-2 from which candidate permeabilizing peptides will be screened and validated for use within the invention. Following the description and teachings herein, permeabilizing JAM-2 peptides (i.e., peptides that operate to measurably increase mucosal epithelial permeability, e.g., by reducing TER and/or increasing rates of transport of macromolecules across mucosal epithelial cell layers in culture, or across mucosal tissues and/or into selected tissues or physiological compartments of a mammalian subject *in vivo*) will be readily identified and incorporated within the methods and compositions of the invention.

Table 6.Candidate Permeabilizing Peptides of Human JAM-2 (Scanning peptides)

	SEQ ID NO:		SEQ ID NO:
PANEL 1		**PANEL 2**	
AVNLKSSNRT	525	AVNLKSSN	568
PVVQEFESVE	526	RTPVVQEF	569
LSCIITDSQT	527	ESVELSCI	570
SDPRIEWKKI	528	ITDSQTSD	571
QDEQTTYVFF	529	PRIEWKKI	572
DNKIQGDLAG	530	QDEQTTYV	573
RAEILGKTSL	531	FFDNKIQG	574
KIWNVTRRDS	532	DLAGRAEI	575
ALYRCEVVAR	533	LGKTSLKI	576
NDRKEIDEIV	534	WNVTRRDS	577
IELTVQVKPV	535	ALYRCEVV	578
TPVCRVPKAV	536	ARNDRKEI	579
PVGKMATLHC	537	DEIVIELT	580
QESEGHPRPH	538	VQVKPVTP	581
YSWYRNDVPL	539	VCRVPKAV	582
PTDSRANPRF	540	PVGKMATL	583
RNSSFHLNSE	541	HCQESEGH	584
TGTLVFTAVH	542	PRPHYSWY	585
KDDSGQYYCI	543	RNDVPLPT	586
ASNDAGSARC	544	DSRANPRF	587
EEQEMEVYDLN	545	RNSSFHLN	588
PANEL 3		SETGTLVF	589
		TAVHKDDS	590
AVNLK	546	GQYYCIAS	591
SSNRTPVVQE	547	NDAGSARC	592
FESVELSCII	548	EEQEMEVY	593
TDSQTSDPRI	549	DLN	594
EWKKIQDEQT	550	**PANEL 4**	
TYVFFDNKIQ	551		
GDLAGRAEIL	552	AVNL	595
GKTSLKIWNV	553	KSSNRTPV	596
TRRDSALYRC	554	VQEFESVE	597
EVVARNDRKE	555	LSCIITDS	598
IDEIVIELTV	556	QTSDPRIE	599
QVKPVTPVCR	557	WKKIQDEQ	600
VPKAVPVGKM	558	TTYVFFDN	601
ATLHCQESEG	559	KIQGDLAG	602
HPRPHYSWYR	560	RAEILGKT	603
NDVPLPTDSR	561	SLKIWNVT	604
ANPRFRNSSF	562	RRDSALYR	605
HLNSETGTLV	563	CEVVARND	606
FTAVHKDDSG	564	RKEIDEIV	607
QYYCIASNDA	565	IELTVQVK	608
GSARCEEQEM	566	PVTPVCRV	609
EVYDLN	567	PKAVPVGK	610
		MATLHCQE	611
		SEGHPRPH	612
		YSWYRNDV	613
		PLPTDSRA	614
		NPRFRNSS	615
		FHLNSETG	616
		TLVFTAVH	617
		KDDSGQYY	618
		CIASNDAG	619
		SARCEEQE	620
		MEVYDLN	621

Human JAM-3 is a polypeptide of 298 amino acids having a predicted extracellular domain from amino acids 28 to 236. The full-length sequence of JAM-3 is provided and the extracellular domain is underlined in Figure 3. Table 7 presents four panels of scanning peptides from the extracellular domain of human JAM-3 from which candidate permeabilizing peptides will be screened and validated for use within the invention. Following the description and teachings herein, permeabilizing JAM-3 peptides will be readily identified and incorporated within the methods and compositions of the invention.

Table 7. Exemplary Permeabilizing Peptides of Human JAM-3 (Scanning peptides)

	SEQ ID NO:		SEQ ID NO:
PANEL 1		**PANEL 2**	
GFSAPKDQQV	622	GFSAPKDQ	665
VTAVEYQEAI	623	QVVTAVEY	666
LACKTPKKTV	624	QEAILACK	667
SSRLEWKKLG	625	TPKKTVSS	668
RSVSFVYYQQ	626	RLEWKKLG	669
TLQGDFKNRA	627	RSVSFVYY	670
EMIDFNIRIK	628	QQTLQGDF	671
NVTRSDAGKY	629	KNRAEMID	672
RCEVSAPSEQ	630	FNIRIKNV	673
GQNLEEDTVT	631	TRSDAGKY	674
LEVLVAPAVP	632	RCEVSAPS	675
SCEVPSSALS	633	EQGQNLEE	676
GTVVELRCQD	634	DTVTLEVL	677
KEGNPAPEYT	635	VAPAVPSC	678
WFKDGIRLLE	636	EVPSSALS	679
NPRLGSQSTN	637	GTVVELRC	680
SSYTMNTKTG	638	QDKEGNPA	681
TLQFNTVSKL	639	PEYTWFKD	682
DTGEYSCEAR	640	GIRLLENP	683
NSVGYRRCPG	641	RLGSQSTN	684
KRMQVDDLN	642	SSYTMNTK	685
		TGTLQFNT	686
PANEL 3		VSKLDTGE	687
		YSCEARNS	688
GFSAP	643	VGYRRCPG	689
KDQQVVTAVE	644	KRMQVDDLN	642
YQEAILACKT	645		
PKKTVSSRLE	646	**PANEL 4**	
WKKLGRSVSF	647		
VYYQQTLQGD	648	GFSA	690
FKNRAEMIDF	649	PKDQQVVT	691
NIRIKNVTRS	650	AVEYQEAI	692
DAGKYRCEVS	651	LACKTPKK	693
APSEQGQNLE	652	TVSSRLEW	694
EDTVTLEVLV	653	KKLGRSVS	695
APAVPSCEVP	654	FVYYQQTL	696
SSALSGTVVE	655	QGDFKNRA	697
LRCQDKEGNP	656	EMIDFNIR	698
APEYTWFKDG	657	IKNVTRSD	699
IRLLENPRLG	658	AGKYRCEV	700
SQSTNSSYTM	659	SAPSEQGQ	701
NTKTGTLQFN	660	NLEEDTVT	702
TVSKLDTGEY	661	LEVLVAPA	703
SCEARNSVGY	662	VPSCEVPS	704
RRCPGKRMQV	663	SALSGTVV	705
DDLN	664	ELRCQDKE	706
		GNPAPEYT	707
		WFKDGIRL	708
		LENPRLGS	709
		QSTNSSYT	710
		MNTKTGTL	711
		QFNTVSKL	712
		DTGEYSCE	713
		ARNSVGYR	714
		RCPGKRMQ	715
		VDDLN	716

Human claudin-1 is a polypeptide of 211 amino acids having three predicted extracellular domains. Exemplary candidate permeabilizing peptides, having a length of between about 4-25 amino acids and comprising a portion of a claudin-1 extracellular domain, are shown in Table 8, below. The two claudin-1 extracellular domains are underlined in Figure 4. Table 8 presents two panels of scanning peptides from the first extracellular domain of human claudin-1, and one exemplary panel of scanning peptides from the second extracellular domain of human claudin-1, from which candidate permeabilizing peptides will be screened and validated for use within the invention. By these methods, the above-noted exemplary permeabilizing peptides of human claudin-1 (YAGDNIVTAQ (SEQ ID NO: 717) and MTPVNARYEF (SEQ ID NO: 718)) were identified. Following the description and teachings herein, additional permeabilizing peptides (i.e., peptides that operate to measurably increase mucosal epithelial permeability, e.g., by reducing TER and/or increasing rates of transport of macromolecules across mucosal epithelial cell layers in culture, or across mucosal tissues and/or into selected tissues or physiological compartments of a mammalian subject *in vivo*) will be readily identified and incorporated within the methods and compositions of the invention.

Human claudin-2 is a polypeptide of 230 amino acids having three predicted extracellular domains. The two extracellular domains are underlined in Figure 5. Exemplary permeabilizing peptides between 4 and 25 amino acids and comprising a portion of a claudin-2 extracellular domain, are shown in Table 8, below. Table 8 presents two panels of scanning peptides from the first extracellular domain of human claudin-2, and one exemplary panel of scanning peptides from the second extracellular domain of human claudin-2, from which candidate permeabilizing peptides will be screened and validated for use within the invention. By these methods, the above-noted exemplary permeabilizing peptides of human claudin-2 (GILRDFYSPL (SEQ ID NO: 53), VPDSMKFEIG (SEQ ID NO: 60), DIYSTLLGLP (SEQ ID NO: 55), and GFSLGLWMEC (SEQ ID NO: 56)) were identified. Following the description and teachings herein, additional permeabilizing peptides of claudin-2 (i.e., peptides that operate to measurably increase mucosal epithelial permeability will be readily identified and incorporated within the methods and compositions of the invention.

Human claudin-3 is a polypeptide of 220 amino acids. The two extracellular domains are underlined in Figure 6. Exemplary permeabilizing peptides between 4

and 25 amino acids and comprising a portion of a claudin-3 extracellular domain, are shown in Table 8, below. Table 8 presents two panels of scanning peptides from the first extracellular domain of human claudin-3, and one exemplary panel of scanning peptides from the second extracellular domain of human claudin-3, from which candidate permeabilizing peptides will be screened and validated for use within the invention. By these methods, the above-noted exemplary permeabilizing peptides of human claudin-3 (NTIIRDFYNP (SEQ ID NO: 54) and VVPEAQKREM (SEQ ID NO: 63)) were identified. Following the description and teachings herein, additional permeabilizing peptides of claudin-3 (i.e., peptides that operate to measurably increase mucosal epithelial permeability will be readily identified and incorporated within the methods and compositions of the invention.

Human claudin-4 is a polypeptide of 209 amino acids. The extracellular domains are underlined in Figure 7. Exemplary permeabilizing peptides between 4 and 25 amino acids and comprising a portion of a claudin-4 extracellular domain, are shown in Table 8, below. Table 8 presents two panels of scanning peptides from the first extracellular domain of human claudin-4, and one exemplary panel of scanning peptides from the second extracellular domain of human claudin-4, from which candidate permeabilizing peptides will be screened and validated for use within the invention. By these methods, the above-noted exemplary permeabilizing peptides of human claudin-4 (VASGQKREMG (SEQ ID NO: 59) and NIIQDFYNPL (SEQ ID NO: 61)) were identified. Following the description and teachings herein, additional permeabilizing peptides of claudin-4 will be readily identified and incorporated within the methods and compositions of the invention.

Human claudin-5 is a polypeptide of 218 amino acids. The extracellular domains are underlined in Figure 8. Exemplary permeabilizing peptides between 4 and 25 amino acids and comprising a portion of a claudin-5 extracellular domain, are shown in Table 8, below. Table 8 presents two panels of scanning peptides from the first extracellular domain of human claudin-5, and one exemplary panel of scanning peptides from the second extracellular domain of human claudin-5, from which candidate permeabilizing peptides will be screened and validated for use within the invention. By these methods, the above-noted exemplary permeabilizing peptide of human claudin-5 (VPVSQKYELG (SEQ ID NO: 62)) was identified. Following the description and teachings herein, additional permeabilizing peptides of claudin-5 will

be readily identified and incorporated within the methods and compositions of the invention.

Human claudin-6 is a polypeptide of 220 amino acids. The extracellular domains are underlined in Figure 9. Exemplary permeabilizing peptides between 4 and 25 amino acids and comprising a portion of a claudin-6 extracellular domain, are shown in Table 8, below. Table 8 presents two panels of scanning peptides from the first extracellular domain of human claudin-6, and one exemplary panel of scanning peptides from the second extracellular domain of human claudin-6, from which candidate permeabilizing peptides will be screened and validated for use within the invention.

Human claudin-7 is a polypeptide of 211 amino acids. The extracellular domains are underlined in Figure 10. Exemplary permeabilizing peptides between 4 and 25 amino acids and comprising a portion of a claudin-7 extracellular domain, are shown in Table 8, below. Table 8 presents two panels of scanning peptides from the first extracellular domain of human claudin-7, and one exemplary panel of scanning peptides from the second extracellular domain of human claudin-7, from which candidate permeabilizing peptides will be screened and validated for use within the invention.

Human claudin-8 is a polypeptide of 225 amino acids. The extracellular domains are underlined in Figure 11. Exemplary permeabilizing peptides between 4 and 25 amino acids and comprising a portion of a claudin-8 extracellular domain, are shown in Table 8, below. Table 8 presents two panels of scanning peptides from the first extracellular domain of human claudin-8, and one exemplary panel of scanning peptides from the second extracellular domain of human claudin-8, from which candidate permeabilizing peptides will be screened and validated for use within the invention.

Human claudin-9 is a polypeptide of 217 amino acids. The extracellular domains are underlined in Figure 12. Exemplary permeabilizing peptides between 4 and 25 amino acids and comprising a portion of a claudin-9 extracellular domain, are shown in Table 8, below. Table 8 presents two panels of scanning peptides from the first extracellular domain of human claudin-9, and one exemplary panel of scanning peptides from the second extracellular domain of human claudin-9, from which candidate permeabilizing peptides will be screened and validated for use within the invention.

Human claudin-10 is a polypeptide of 228 amino acids. The extracellular domains are underlined in Figure 13. Exemplary permeabilizing peptides between 4 and 25 amino acids and comprising a portion of a claudin-10 extracellular domain, are shown in Table 8, below. Table 8 presents two panels of scanning peptides from the first extracellular domain of human claudin-10, and one exemplary panel of scanning peptides from the second extracellular domain of human claudin-10, from which candidate permeabilizing peptides will be screened and validated for use within the invention.

Table 8. Exemplary Permeabilizing Peptides of Human Claudins 1-10 (Scanning peptides)

Human Claudin-1	SEQ ID NO:	Human Claudin-4	SEQ ID NO:
PANEL 1; ECD 1		**PANEL 1; ECD 1**	
RIYSYAGDNI	719	RVTAFIGSNI	747
VTAQAMYEGL	720	VTSQTIWEGL	748
WMSCVSQSTG	721	WMNCVVQSTG	749
QIQCKVFDSL	722	QMQCKVYDSL	750
LNLSSTLQATR	723	LALPQDLQAAR	751
PANEL 2; ECD 1		**PANEL 2; ECD 1**	
RIYSY	724	RVTAF	752
AGDNIVTAQA	725	IGSNIVTSQT	753
MYEGLWMSCV	726	IWEGLWMNCV	754
SQSTGQIQCK	727	VQSTGQMQCK	755
VFDSLLNLSS	728	VYDSLLALPQ	756
TLQATR	729	DLQAAR	757
PANEL 1; ECD 2		**PANEL 1; ECD 2**	
QEFYDPMT	730	QDFYN.	758
PVNARYE	731	PLV	759
QEFYDPMTPVN	732	ASGQKRE	
ARYE	733		
Human Claudin-2	**SEQ ID NO:**	**Human Claudin-5**	**SEQ ID NO:**
PANEL 1; ECD 1		**PANEL 1; ECD 1**	
KTSSYVGASI	734	QVTAFLDHNI	760
VTAVGFSKGL	735	VTAQTTWKGL	761
WMECATHSTG	736	WMSCVVQSTG	749
ITQCDIYSTL	737	HMQCKVYDSV	762
LGLPADIQAAQ	738	LALSTEVQAAR	763
PANEL 2; ECD 1		**PANEL 2; ECD 1**	
KTSSY	739	QVTAF	764
VGASIVTAVG	740	LDHNIVTAQT	765
FSKGLWMECA	741	TWKGLWMSCV	766
THSTGITQCD	742	VQSTGHMQCK	767
IYSTLLGLPA	743	VYDSVLALST	768
DIQAAQ	744	EVQAAR	769

PANEL 1; ECD 2		**PANEL 1; ECD 2**	
RDFYSPL	745	REFYDPSV	770
VPDSMKFE	746	PVSQKYE	771
Human Claudin-3	**SEQ ID NO:**	**Human Claudin-6**	**SEQ ID NO:**
PANEL 1; ECD 1		**PANEL 1; ECD 1**	
RVSAFIGSNI	772	KVTAFIGNSI	777
ITSQNIWEGL	773	VVAQVVWEGL	778
WMNCVVQSTG	749	WMSCVVQSTG	749
QMQCKVYDSL	750	QMQCKVYDSL	750
LALPQDLQAAR	751	LALPQDLQAAR	751
PANEL 2; ECD 1		**PANEL 2; ECD 1**	
RVSAF	774	KVTAF	779
IGSNIITSQN	868	IGNSIVVAQV	780
IWEGLWMNCV	754	VWEGLWMSCV	781
VQSTGQMQCK	755	VQSTGQMQCK	755
VYDSLLALPQ	756	VYDSLLALPQ	756
DLQAAR	757	DLQAAR	757
PANEL 1; ECD 2		**PANEL 1; ECD 2**	
RDFYNPVV	775	RDFYNPLV	782
PEAQKRE	776	AEAQKRE	783

Table 8. Exemplary Permeabilizing Peptides of Human Claudin (Scanning peptides)(continued)

Human Claudin-7	SEQ ID NO:	Human Claudin-9	SEQ ID NO:
PANEL 1; ECD 1		**PANEL 1; ECD 1**	
QMSSYAGDNI	784	KVTAFIGNSI	809
ITAQAMYKGL	785	VVAQVVWEGL	778
WMDCVTQSTG	786	WMSCVVQSTG	749
MMSCKMYDSV	787	QMQCKVYDSL	750
LALSAALQATR	788	LALPQDLQAAR	751
PANEL 2; ECD 1		**PANEL 2; ECD 1**	
QMSSY	789	KVTAF	779
AGDNIITAQA	790	IGNSIVVAQV	780
MYKGLWMDCV	791	VWEGLWMSCV	781
TQSTGMMSCK	792	VQSTGQMQCK	755
MYDSVLALSA	793	VYDSLLALPQ	756
ALQATR	794	DLQAAR	757
PANEL 1; ECD 2		**PANEL 1; ECD 2**	
TDFYNPLI	795	QDFYNPLV	758
PTNIKYE	796	AEALKRE	810
Human Claudin-8	**SEQ ID NO:**	**Human Claudin-10**	**SEQ ID NO:**
PANEL 1; ECD 1		**PANEL 1; ECD 1**	
RVSAFIENNI	797	KVSTIDGTVI	811
VVFENFWEGL	798	TTATYWANLW	812
WMNCVRQANI	799	KACVTDSTGV	813
RMQCKIYDSL	800	SNCKDFPSML	814
LALSPDLQAAR	801	ALDGYIQACR	815
PANEL 2; ECD 1		**PANEL 2; ECD 1**	
RVSAF	802	KVSTI	816

IENNIVVFEN	**803**	DGTVITTATY	**817**
FWEGLWMNCV	**804**	WANLWKACVT	**818**
RQANIRMQCK	**805**	DSTGVSNCKD	**819**
IYDSLLALSP	**806**	FPSMLALDGY	**820**
DLQAAR	**807**	IQACR	**821**
PANEL 1; ECD 2		**PANEL 1; ECD 2**	
RDFYNSIV	**807**	EFFDPLF	**822**
NVAQKRE	**808**	VEQKYE	**823**

Human occludin is a polypeptide of 522 amino acids. The two extracellular domains of human occludin are underlined in Figure 14. Exemplary candidate permeabilizing peptides, having a length of between about 4-25 amino acids and comprising a portion of a human occludin extracellular domain, are shown in Table 9, below. The two occludin extracellular domains are underlined in Figure 14. Table 9 presents two panels of scanning peptides from each of the first and second extracellular domains of human occludin, from which candidate permeabilizing peptides will be screened and validated for use within the invention. By these methods, the above-noted exemplary permeabilizing peptides of human occludin (GVNPTAQSS (SEQ ID NO: 33), GSLYGSQIY (SEQ ID NO: 34), AATGLYVDQ (SEQ ID NO: 32), ALCNQFYTP (SEQ ID NO: 35), and YLYHYCVVD (SEQ ID NO: 36)) were identified. Following the description and teachings herein, additional permeabilizing peptides (i.e., peptides that operate to measurably increase mucosal epithelial permeability, e.g., by reducing TER and/or increasing rates of transport of macromolecules across mucosal epithelial cell layers in culture, or across mucosal tissues and/or into selected tissues or physiological compartments of a mammalian subject *in vivo*) of human occludin will be readily identified and incorporated within the methods and compositions of the invention.

Table 9. Exemplary Permeabilizing Peptides of Human Occludin (Scanning peptides)

	SEQ ID NO:
PANEL 1; ECD 1	
DRGYGTSLLG	824
GSVGYPYGGS	825
GFGSYGSGYG	826
YGYGYGYGYG	827
GYTDPR	828
PANEL 2; ECD 1	
DRGYG	829
TSLLGGSVGY	830
PYGGSGFGSY	831
GSGYGYGYGY	832
GYGYGGYTDPR	833
PANEL 1; ECD 2	
GVNPTAQSSG	834
SLYGSQIYAL	835
CNQFYTPAAT	836
GLYVDQYLYH	837
YCVVDPQE	838
PANEL 2; ECD 2	
GVNPT	839
AQSSGSLYGS	840
QIYALCNQFY	841
TPAATGLYVD	842
QYLYHYCVVD	843
PQE	844

In an exemplary embodiment, the uptake of intranasally administered a biologically active agent, for example, interferon-β, in combination with a mucosal delivery-enhancing effective amount of a permeabilizing peptide into the blood serum of a mammalian subject is determined. The permeabilizing peptide reversibly enhances mucosal epithelial paracellular transport by modulating epithelial junctional structure and/or physiology in a mammalian subject. The permeabilizing peptide generally effectively inhibits homotypic binding of an epithelial membrane adhesive protein selected from a junctional adhesion molecule (JAM), occludin, or claudin protein.

Pharmacokinetic data for intranasal delivery of a biologically active agent, for example, interferon-β-1a, in a pharmaceutical formulation comprising a permeabilizing peptide of JAM-1, claudin-2, or occludin of the present invention can be determined by a variety of methods. In an exemplary embodiment, maximum concentration of interferon-β in the blood serum (C_{max}) at 3 hours following intranasal delivery of the pharmaceutical formulation of the present invention is measured and will be approximately 5.0 IU/mL or greater, typically 6.0 IU/mL or greater, or 10.0 IU/mL or greater.

Time to maximum serum concentration of interferon-β in the blood serum (t_{max}) is accelerated by the formulations and methods of the present invention compared to subcutaneous or intramuscular delivery of interferon-β-1a. In an exemplary embodiment, t_{max} for intranasal delivery of the formulation of the present invention is approximately 0.4 hours or less, typically 0.3 hours or less.

Within other detailed aspects of the invention, bioavailability of interferon-β following administration in accordance with the methods and compositions of the invention is determined by measuring interferon-β "pharmacokinetic markers". As used herein, pharmacokinetic markers include any accepted biological marker that is detectable in an *in vitro* or *in vivo* system useful for modeling pharmacokinetics of mucosal delivery of one or more interferon-β compounds, or other biologically active agent(s) disclosed herein, wherein levels of the marker(s) detected at a desired target site following administration of the interferon-β compound(s) according to the methods and formulations herein, provide a reasonably correlative estimate of the level(s) of the interferon-β compound(s) delivered to the target site. Among many art-accepted markers in this context are substances induced at the target site by adminstration of the interferon-β compound(s) or orther biologically active agent(s). For example, nasal mucosal delivery of an effective amount of one or more interferon-β compounds according to the invention stimulates an immunologic response in the subject measurable by production of pharmacokinetic markers that include, but are not limited to, neopterin, β_2-microglobulin, and 2', 5'-oligoadenylate synthetase.

Art-accepted pharmacokinetic markers for interferon-β, for example, serum β-2 microglobulin, serum neopterin or serum 2',5'-oligoadenylate synthetase, may be measured following administration, e.g., as measured by peak blood plasma levels

(C_{max}) of the marker(s) in blood serum, CNS tissues or compartments, CSF or in another selected physiological compartment or target tissue. These and other such marker data are accepted in the art as reasonably correlated with pharmacokinetics of interferon-β compounds that may be undetectable directly *in vivo*. In certain aspects, enhanced bioavailability of interferon-β as measured by interferon-β markers will be demonstrated by, for example, a correlated C_{max} for serum β-2 microglobulin of approximately 1.7 mg/ml of blood plasma or CSF, or approximately 2.0 mg/ml of blood plasma or CSF, or approximately 4.0 mg/ml or greater of blood plasma or CSF. C_{max} for serum neopterin of approximately 8 nmol/l of blood plasma or CSF, approximately 10 nmol/l of blood plasma or CSF, approximately 20 nmol/l of blood plasma or CSF, approximately 30 nmol/l of blood plasma or CSF, or approximately 40 nmol/l or greater of blood plasma or CSF.

Within further detailed aspects, the pharmaceutical composition as disclosed herein following mucosal administration to said subject yields a peak concentration (C_{max}) for pharmacological markers, neopterin or β2-microglobulin in the blood plasma or CNS tissue or fluid of the subject that is typically 25% or greater, or 75% or greater, or 150% or greater, as compared to a peak concentration of neopterin or β2-microglobulin in blood plasma or CNS tissue or fluid following intramuscular injection of an equivalent concentration or dose of interferon-β to said subject, intranasal delivery of interferon-β alone, and/or mucosal delivery of interferon-β using previously-described methods and formulations.

Within other detailed aspects of the invention, bioavailability of interferon-β will be determined by measuring interferon-β pharmacokinetic markers, for example, serum β-2 microglobulin or serum neopterin, to estimate area under the concentration curve (AUC) for the marker(s) in blood serum, CNS, CSF or in another selected physiological compartment or target tissue. Bioavailability of interferon-β as determined by interferon-β markers in this context will be, for example, $AUC_{0\text{-}96\,hr}$ for serum β-2 microglobulin of approximately 200 μIU•hr/mL of blood plasma or CSF, $AUC_{0\text{-}96\,hr}$ for β-2 microglobulin up to approximately 500 μIU•hr/mL of blood plasma or CSF, $AUC_{0\text{-}96\,hr}$ for serum neopterin of approximately 200 ng·hr/ml of blood plasma or CSF, $AUC_{0\text{-}96\,hr}$ for serum neopterin up to approximately 500 ng·hr/ml of blood plasma or CSF.

Within further detailed aspects, the pharmaceutical composition as disclosed herein following mucosal administration to said subject yields area under the concentration curve ($AUC_{0\text{-}96\ hr}$) for pharmacological markers, neopterin or β2-microglobulin, in the blood plasma or CNS tissue or fluid of the subject that is typically 25% or greater, or 75% or greater, or 150% or greater, as compared to an $AUC_{0\text{-}96\ hr}$ for neopterin or β2-microglobulin in blood plasma or CNS tissue or fluid following intramuscular injection of an equivalent concentration or dose of interferon-β to said subject, intranasal delivery of interferon-β alone, and/or mucosal delivery of interferon-β using previously-described methods and formulations.

Within yet additional detailed aspects of the invention, bioavailability of interferon-β pharmacokinetic markers, for example, serum β-2 microglobulin or serum neopterin, achieved by the methods and formulations herein is measured by time to maximal concentration (t_{max}) in blood serum, CNS, CSF or in another selected physiological compartment or target tissue. t_{max} for serum β-2 microglobulin will be, for example, about 45 hours or less, typically 35 hours or less, or typically 25 hours or less following intranasal administration of interferon-β by methods and formulations described herein. t_{max} for serum neopterin will be, for example, about 40 hours or less, typically 30 hours or less, or typically 25 hours or less following intranasal administration of interferon-β by methods and formulations described herein.

Within further detailed aspects, the pharmaceutical composition as disclosed herein following mucosal administration to said subject yields a time to maximal plasma concentration (t_{max}) for pharmacological markers, neopterin or β_2-microglobulin, in a blood plasma or CNS tissue or fluid of the subject that is typically between about 25 to 45 hours, or between about 25 to 35 hours.

The results indicate that significant plasma levels (C_{max}) of interferon-β are achieved following intranasal administration of a pharmaceutical formulation of interferon-β in combination with one or more intranasal delivery-enhancing agents, e.g., permeabilizing peptides, JAM, claudin, or occludin of the present invention. The time to maximum serum concentration (t_{max}) by intranasal delivery is accelerated at least 5- to 10-fold to achieve similar blood plasma levels (C_{max}) when compared to subcutaneous or intramuscular injection. The rate of delivery of interferon-β by intranasal administration of pharmaceutical formulations of the present invention (as

measured by C_{max} and t_{max}) is significantly increased when compared to the rate of delivery by intramuscular or subcutaneous injection of interferon-β.

Intranasal administration of a pharmaceutical formulation comprising permeabilizing peptide of the present invention, e.g., JAM, claudin, or occludin peptide, 10 minutes, 20 minutes or 30 minutes prior to intranasal administration of an interferon-β formulation provides advantages to improve delivery (C_{max} and t_{max}) of interferon-β to the CNS or blood serum by 5 to 10 percent, 10 to 15 percent, or 15 to 20 percent compared to intranasal administration of interferon-β formulation alone.

The potential to deliver and maintain consistent therapeutic blood levels and CNS levels of interferon-β by pharmaceutical formulations comprising permeabilizing peptide of the present invention provide a distinct advantage over existing formulations for intramuscular or subcutaneous administration. A distinct advantage exists for maintaining consistent therapeutic blood levels and CNS levels of interferon-β by repeated intranasal administration within a 0.5 to 1 hour time frame in which maximum concentration in the blood serum is achieved, as compared to subcutaneous administration which requires 4 hours or longer to reach maximum concentration in the blood serum. Pharmacodynamic markers of interferon-β activity indicate a maximum concentration of IFN-β markers, neopterin and β_2-microglobulin are achieved in approximately 45 hours or less, typically in 30 hours or less, or typically 22 hours or less following intranasal administration of interferon-β by pharmaceutical formulations comprising permeabilizing peptide of the present invention.

BIOLOGICALLY ACTIVE AGENTS

The methods and compositions of the present invention are directed toward enhancing mucosal, e.g., intranasal, delivery of a broad spectrum of biologically active agents to achieve therapeutic, prophylactic or other desired physiological results in mammalian subjects. As used herein, the term "biologically active agent" encompasses any substance that produces a physiological response when mucosally administered to a mammalian subject according to the methods and compositions herein. Useful biologically active agents in this context include therapeutic or prophylactic agents applied in all major fields of clinical medicine, as well as

nutrients, cofactors, enzymes (endogenous or foreign), antioxidants, and the like. Thus, the biologically active agent may be water-soluble or water-insoluble, and may include higher molecular weight proteins, peptides, carbohydrates, glycoproteins, lipids, and/or glycolipids, nucleosides, polynucleotides, and other active agents.

Useful pharmaceutical agents within the methods and compositions of the invention include drugs and macromolecular therapeutic or prophylactic agents embracing a wide spectrum of compounds, including small molecule drugs, peptides, proteins, and vaccine agents. Exemplary pharmaceutical agents for use within the invention are biologically active for treatment or prophylaxis of a selected disease or condition in the subject. Biological activity in this context can be determined as any significant (i.e., measurable, statistically significant) effect on a physiological parameter, marker, or clinical symptom associated with a subject disease or condition, as evaluated by an appropriate *in vitro* or *in vivo* assay system involving actual patients, cell cultures, sample assays, or acceptable animal models.

The methods and compositions of the invention provide unexpected advantages for treatment of diseases and other conditions in mammalian subjects, which advantages are mediated, for example, by providing enhanced speed, duration, fidelity or control of mucosal delivery of therapeutic and prophylactic compounds to reach selected physiological compartments in the subject (e.g., into or across the nasal mucosa, into the systemic circulation or central nervous system (CNS), or to any selected target organ, tissue, fluid or cellular or extracellular compartment within the subject).

In various exemplary embodiments, the methods and compositions of the invention may incorporate one or more biologically active agent(s) selected from:

opiods or opiod antagonists, such as morphine, hydromorphone, oxymorphone, lovorphanol, levallorphan, codeine, nalmefene, nalorphine, nalozone, naltrexone, buprenorphine, butorphanol, and nalbufine;

corticosterones, such as cortisone, hydrocortisone, fludrocortisone, prednisone, prednisolone, methylprednisolone, triamcinolone, dexamethoasone, betamethoasone, paramethosone, and fluocinolone;

other anti-inflammatories, such as colchicine, ibuprofen, indomethacin, and piroxicam; anti-viral agents such as acyclovir, ribavarin, trifluorothyridine, Ara-A (Arabinofuranosyladenine), acylguanosine, nordeoxyguanosine, azidothymidine, dideoxyadenosine, and dideoxycytidine; antiandrogens such as spironolactone;

androgens, such as testosterone;

estrogens, such as estradiol;

progestins;

muscle relaxants, such as papaverine;

vasodilators, such as nitroglycerin, vasoactive intestinal peptide and calcitonin related gene peptide;

antihistamines, such as cyproheptadine;

agents with histamine receptor site blocking activity, such as doxepin, imipramine, and cimetidine;

antitussives, such as dextromethorphan; neuroleptics such as clozaril; antiarrhythmics;

antiepileptics;

enzymes, such as superoxide dismutase and neuroenkephalinase;

anti-fungal agents, such as amphotericin B, griseofulvin, miconazole, ketoconazole, tioconazol, itraconazole, and fluconazole;

antibacterials, such as penicillins, cephalosporins, tetracyclines, aminoglucosides, erythromicin, gentamicins, polymyxin B;

anti-cancer agents, such as 5-fluorouracil, bleomycin, methotrexate, and hydroxyurea, dideoxyinosine, floxuridine, 6-mercaptopurine, doxorubicin, daunorubicin, I-darubicin, taxol and paclitaxel (optionally provided in a bimodal emulsion, e.g., as described in U.S. Patent Application Serial No. 09/631,246, filed by Quay on August 02, 2000);

antioxidants, such as tocopherols, retinoids, carotenoids, ubiquinones, metal chelators, and phytic acid;

antiarrhythmic agents, such as quinidine; and

antihypertensive agents such as prazosin, verapamil, nifedipine, and diltiazem; analgesics such as acetaminophen and aspirin;

monoclonal and polyclonal antibodies, including humanized antibodies, and antibody fragments;

anti-sense oligonucleotides; and

RNA, DNA and viral vectors comprising genes encoding therapeutic peptides and proteins.

In addition to these exemplary classes and species of active agents, the methods and compositions of the invention embrace any physiologically active agent,

as well as any combination of multiple active agents, described above or elsewhere herein or otherwise known in the art, that is individually or combinatorially effective within the methods and compositions of the invention for treatment or prevention of a selected disease or condition in a mammalian subject (see, Physicians' Desk Reference, published by Medical Economics Company, a division of Litton Industries, Inc).

Regardless of the class of compound employed, the biologically active agent for use within the invention will be present in the compositions and methods of the invention in an amount sufficient to provide the desired physiological effect with no significant, unacceptable toxicity or other adverse side effects to the subject. The appropriate dosage levels of all biologically active agents will be readily determined without undue experimentation by the skilled artisan. Because the methods and compositions of the invention provide for enhanced delivery of the biologically active agent(s), dosage levels significantly lower than conventional dosage levels may be used with success. In general, the active substance will be present in the composition in an amount of from about 0.01% to about 50%, often between about 0.1 % to about 20%, and commonly between about 1.0% to 5% or 10% by weight of the total intranasal formulation depending upon the particular substance employed.

PEPTIDE AND PROTEIN AGENTS

The value of biologically active peptides and proteins in medicine has been long recognized in the art. Peptides and proteins are contemplated as potentially ideal therapeutics, due to their specificity of action, their effectiveness *in vivo* at relatively low concentrations, and their rapid catalytic activity. For many years, the lack of industrial manufacturing processes for peptides and proteins limited their use as therapeutic agents. However, in recent years the biotechnology and genetic engineering fields have advanced dramatically, making possible the availability of numerous such therapeutic agents for clinical use (see, e.g., Swann, Pharm. Res. 16:826-834, 1998).

Unfortunately, proteins possess characteristics such as low bioavailability and chemical stability problems (Putney et al., Nature Biotech. 16:153-157, 1998) that may limit their use for treatment of certain diseases. The delivery of peptides and

proteins to the body is usually performed by frequent injections. This results in a rapid increase and subsequent rapid decrease of the blood serum concentration levels that could lead to the appearance of side effects. Therefore, the major challenge in this field is to design a system capable of maintaining an effective concentration of the active agent for an effective period at a target tissue or physiological compartment and to minimize the number of doses that have to be administered.

As used herein, the terms biolotically active "peptide" and "protein" include polypeptides of various sizes, and do not limit the invention to amino acid polymers of any particular size. Peptides from as small as a few amino acids in length, to proteins of any size, as well as peptide-peptide, protein-protein fusions and protein-peptide fusions, are encompassed by the present invention, so long as the protein or peptide is biologically active in the context of eliciting a specific physiological, immunological, therapeutic, or prophylactic effect or response.

Numerous peptides and proteins have been isolated and developed for use in, for example, treatment of conditions associated with a protein deficiency (e.g., human growth hormone, insulin); enhancement of immune responses (e.g., antibodies, cytokines); treatment of cancer (e.g., cytokines, L-asparaginase, superoxide dismutase, monoclonal antibodies); treatment of conditions associated with excessive or inappropriate enzymatic activity (e.g., inhibition of elastase with alpha-1-antitrypsin, regulation of blood clotting with antithrombin-III); blood replacement therapy (e.g., hemoglobin); treatment of endotoxic shock (e.g., bactericidal-permeability increasing (BPI) protein); and wound healing (e.g., growth factors, erythropoietin). The foregoing examples are only representative of the vast possibilities in the emerging field of peptide and protein therapy.

The formulation and delivery of relatively high molecular weight peptide and protein drugs can present certain problems due to their fragile nature when compared to traditional, smaller molecular weight drugs. In order to successfully employ peptides and proteins as pharmaceuticals, it is essential to understand the many delivery and stability issues relevant to their formulation and effective administration. Peptides and proteins undergo a variety of intra- and inter-molecular chemical reactions which can lead to a decline or loss of their effectiveness as pharmaceuticals. These include oxidation, deamidation, beta-elimination, disulfide scrambling, hydrolysis, isopeptide bond formation, and aggregation. In addition to chemical stability, peptides and proteins must often retain their three dimensional structure in

order to maintain biological activity as therapeutic agents. Loss of the native conformation of peptides and proteins often leads not only to a reduction or loss of biological activity, but also to increased susceptibility to further deleterious processes such as covalent or noncovalent aggregation. Furthermore, the formation of protein aggregates leads to other problems relating to parenteral delivery, such as decreased solubility and increased immunogenicity (see, e.g., H. R. Costantino et al., J. Pharm. Sci. 83:1662-1669, 1994).

The instant invention provides novel formulations and coordinate administration methods for enhanced mucosal delivery of biologically active peptides and proteins. Illustrative examples of therapeutic peptides and proteins for use within the invention include, but are not limited to: tissue plasminogen activator (TPA), epidermal growth factor (EGF), fibroblast growth factor (FGF-acidic or basic), platelet derived growth factor (PDGF), transforming growth factor (TGF-alpha or beta), vasoactive intestinal peptide, tumor necrosis factor (TGF), hypothalmic releasing factors, prolactin, thyroid stimulating hormone (TSH), adrenocorticotropic hormone (ACTH), parathyroid hormone (PTH), follicle stimulating hormone (FSF), luteinizing hormone releasing (LHRH), endorphins, glucagon, calcitonin, oxytocin, carbetocin, aldoetecone, enkaphalins, somatostin, somatotropin, somatomedin, gonadotrophin, estrogen, progesterone, testosterone, alpha-melanocyte stimulating hormone, non-naturally occurring opiods, lidocaine, ketoprofen, sufentainil, terbutaline, droperidol, scopolamine, gonadorelin, ciclopirox, olamine, buspirone, calcitonin, cromolyn sodium or midazolam, cyclosporin, lisinopril, captopril, delapril, cimetidine, ranitidine, famotidine, superoxide dismutase, asparaginase, arginase, arginine deaminease, adenosine deaminase ribonuclease, trypsin, chemotrypsin, and papain. Additional examples of useful peptides include, but are not limited to, bombesin, substance P, vasopressin, alpha-globulins, transferrin, fibrinogen, beta-lipoproteins, beta-globulins, prothrombin, ceruloplasmin, alpha$_2$-glycoproteins, alpha$_2$-globulins, fetuin, alpha$_1$-lipoproteins, alpha$_1$-globulins, albumin, prealbumin, and other bioactive proteins and recombinant protein products.

In more detailed aspects of the invention, methods and compositions are provided for enhanced mucosal delivery of specific, biologically active peptide or protein therapeutics to treat (i.e., to eliminate, or reduce the occurrence or severity of symptoms of) an existing disease or condition, or to prevent onset of a disease or condition in a subject identified to be at risk for the subject disease or condition.

Biologically active peptides and proteins that are useful within these aspects of the invention include, but are not limited to hematopoietics; antiinfective agents; antidementia agents; antiviral agents; antitumoral agents; antipyretics; analgesics; antiinflammatory agents; antiulcer agents; antiallergic agents; antidepressants; psychotropic agents; cardiotonics; antiarrythmic agents; vasodilators; antihypertensive agents such as hypotensive diuretics; antidiabetic agents; anticoagulants; cholesterol lowering agents; therapeutic agents for osteoporosis; hormones; antibiotics; vaccines; and the like.

Biologically active peptides and proteins for use within these aspects of the invention include, but are not limited to, cytokines; peptide hormones; growth factors; factors acting on the cardiovascular system; cell adhesion factors; factors acting on the central and peripheral nervous systems; factors acting on humoral electrolytes and hemal organic substances; factors acting on bone and skeleton growth or physiology; factors acting on the gastrointestinal system; factors acting on the kidney and urinary organs; factors acting on the connective tissue and skin; factors acting on the sense organs; factors acting on the immune system; factors acting on the respiratory system; factors acting on the genital organs; and various enzymes.

For example, hormones which may be administered within the methods and compositions of the present invention include androgens, estrogens, prostaglandins, somatotropins, gonadotropins, interleukins, steroids and cytokines.

Vaccines which may be administered within the methods and compositions of the present invention include bacterial and viral vaccines, such as vaccines for hepatitis, influenza, respiratory syncytial virus (RSV), parainfluenza virus (PIV), tuberculosis, canary pox, chicken pox, measles, mumps, rubella, pneumonia, and human immunodeficiency virus (HIV).

Bacterial toxoids which may be administered within the methods and compositions of the present invention include diphtheria, tetanus, pseudonomas and mycobactrium tuberculosis.

Examples of specific cardiovascular or thromobolytic agents for use within the invention include hirugen, hirulos and hirudine.

Antibody reagents that are usefully administered with the present invention include monoclonal antibodies, polyclonal antibodies, humanized antibodies, antibody fragments, fusions and multimers, and immunoglobins.

Exemplary cytokines for use within the methods and compositions of invention include lymphokines, monokines, hematopoietic factors, and the like, for example interleukins (e.g. interleukin 2 through 11), interleukin-1, tumor necrosis factors (e.g. TNF-alpha and beta), and malignant leukocyte inhibitory factor (LIF), granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage stimulating factor (GM-CSF) and macrophage colony stimulating factor (M-CSF).

Examples of peptide and protein factors which act on bone and skeletal metabolism for use within the methods and compositions of the invention include bone GLa peptide, parathyroid hormone and its active fragments, osteostatin, calcitonin (see, e.g., U.S. Patent Application Serial No. 09/686,452, filed by Quay on October 10, 2000) and histone H4-related bone formation and proliferation peptide.

Exemplary growth factors for use within the methods and compositions of the invention include epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF), transforming growth factor (TGF), platelet-derived cell growth factor (PDGF), hepatocyte growth factor (HGF), and the like.

Exemplary peptide hormones for use within the methods and compositions of the invention include luteinizing hormone, luteinizing hormone-releasing hormone (LH-RH), adrenocorticotropic hormone (ACTH), amylin, oxytocin and carbetocin (see, e.g., U.S. Patent Application Serial No. 09/481,058 and U.S. Patent Application Serial No. 09/678,591, filed by Quay on January 11, 2000, and October 03, 2000, respectively), and the like.

With respect to factors acting on the cardiovascular system, exemplary peptides and proteins for use within the methods and compositions of the invention include those which are biologically active to control blood pressure, arteriosclerosis, and other cardiovascular diseases and conditions, exemplified by endothelins, endothelin inhibitors, and endothelin antagonists (see, e.g., EP 436189, EP 457195, EP 496452 and EP 528312), endothelin producing enzyme inhibitors, vasopressin, renin, angiotensin I, angiotensin II, angiotensin III, angiotensin I inhibitor, angiotensin II receptor antagonist, antiarrythmic peptide, and so on.

Exemplary peptide and protein factors acting on the central and peripheral nervous systems for use within the methods and compositions of the invention include opioid peptides (e.g. enkepharins, endorphins, kyotorphins), neurotropic factor (NTF), calcitonin gene-related peptide (CGRP), thyroid hormone releasing hormone (TRH), salts and derivatives of TRH (see, e.g., JP Laid Open No. 50-121273/1975; U.S. Pat.

No. 3,959,247; JP Laid Open No. 52-116465/1977; U.S. Pat. No. 4,100,152), neurotensin, and the like.

Exemplary peptide and protein factors acting on the gastrointestinal system for use within the methods and compositions of the invention include secretin and gastrin.

Exemplary peptide and protein factors acting on humoral electrolytes and hemal organic substances for use within the methods and compositions of the invention include known factors which control hemagglutination, plasma cholesterol level or metal ion concentrations, such as calcitonin, apoprotein E and hirudin

Exemplary cell adhesion factors for use within the methods and compositions of the invention include laminin, and intercellular adhesion molecule 1 (ICAM 1).

Exemplary peptide and protein factors acting on the kidney and urinary tract for use within the methods and compositions of the invention include factors that regulate the function of the kidney, such as urotensin.

Exemplary peptide and protein factors acting on the immune system for use within the methods and compositions of the invention include known factors which modulate inflammation and malignant neoplasms, as well as factors which attack infective microorganisms, such as chemotactic peptides and bradykinins.

The biologically active peptides and proteins for use within the invention further include enzymes of natural origin and recombinant enzymes, which include but are not limited to superoxide dismutase (SOD), asparaginase, kallikreins, and the like.

Biologically active peptides and proteins for use within the invention can be peptides or proteins that are readily absorbed into or across the nasal mucosa, but are more typically absorbed poorly (e.g., into the systemic circulation), or not at all, following conventional intranasal delivery/formulation methods. In the latter case, delivery of the peptides or proteins intranasally fails to elicit a therapeutically or prophylactically effective concentration of the peptide or protein at a target compartment (e.g., the systemic circulation) for activity.

Typically, peptides for use within the invention have a molecular weight in the range of about 100 to 200,000, more commonly within the molecular weight range of about 200 to 100,000, and frequently within the range of about 200 to 50,000.

PEPTIDE AND PROTEIN ANALOGS AND MIMETICS

Included within the definition of biologically active peptides and proteins for use within the invention are natural or synthetic, therapeutically or prophylactically active, peptides (comprised of two or more covalently linked amino acids), proteins, peptide or protein fragments, peptide or protein analogs, and chemically modified derivatives or salts of active peptides or proteins. Often, the peptides or proteins are muteins that are readily obtainable by partial substitution, addition, or deletion of amino acids within a naturally occurring or native (e.g., wild-type, naturally occurring mutant, or allelic variant) peptide or protein sequence. Additionally, biologically active fragments of native peptides or proteins are included. Such mutant derivatives and fragments substantially retain the desired biological activity of the native peptide or proteins. In the case of peptides or proteins having carbohydrate chains, biologically active variants marked by alterations in these carbohydrate species are also included within the invention.

In additional embodiments, peptides or proteins for use within the invention may be modified by addition or conjugation of a synthetic polymer, such as polyethylene glycol, a natural polymer, such as hyaluronic acid, or an optional sugar (e.g. galactose, mannose), sugar chain, or nonpeptide compound. Substances added to the peptide or protein by such modifications may specify or enhance binding to certain receptors or antibodies or otherwise enhance the mucosal delivery, activity, half-life, cell- or tissue-specific targeting, or other beneficial properties of the peptide or protein. For example, such modifications may render the peptide or protein more lipophilic, e.g., such as may be achieved by addition or conjugation of a phospholipid or fatty acid. Further included within the methods and compositions of the invention are peptides and proteins prepared by linkage (e.g., chemical bonding) of two or more peptides, protein fragments or functional domains (e.g., extracellular, transmembrane and cytoplasmic domains, ligand-binding regions, active site domains, immunogenic epitopes, and the like)--for example fusion peptides and proteins recombinantly produced to incorporate the functional elements of a plurality of different peptides or proteins in a single encoded molecule.

Biologically active peptides and proteins for use within the methods and compositions of the invention thus include native or “wild-type” peptides and proteins and naturally occurring variants of these molecules, e.g., naturally occurring allelic variants and mutant proteins. Also included are synthetic, e.g., chemically or

recombinantly engineered, peptides and proteins, as well as peptide and protein "analogs" and chemically modified derivatives, fragments, conjugates, and polymers of naturally occurring peptides and proteins. As used herein, the term peptide or protein "analog" is meant to include modified peptides and proteins incorporating one or more amino acid substitutions, insertions, rearrangements or deletions as compared to a native amino acid sequence of a selected peptide or protein, or of a binding domain, fragment, immunogenic epitope, or structural motif, of a selected peptide or protein. Peptide and protein analogs thus modified exhibit substantially conserved biological activity comparable to that of a corresponding native peptide or protein, which means activity (e.g., specific binding to a JAM, occludin or claudin protein, or to a cell expressing such a protein, specific ligand or receptor binding activity, etc.) levels of at least 50%, typically at least 75%, often 85%-95% or greater, compared to activity levels of a corresponding native protein or peptide.

For purposes of the present invention, the term biologically active peptide or protein "analog" further includes derivatives or synthetic variants of a native peptide or protein, such as amino and/or carboxyl terminal deletions and fusions, as well as intrasequence insertions, substitutions or deletions of single or multiple amino acids. Insertional amino acid sequence variants are those in which one or more amino acid residues are introduced into a predetermined site in the protein. Random insertion is also possible with suitable screening of the resulting product. Deletional variants are characterized by removal of one or more amino acids from the sequence. Substitutional amino acid variants are those in which at least one residue in the sequence has been removed and a different residue inserted in its place.

Where a native peptide or protein is modified by amino acid substitution, amino acids are generally replaced by other amino acids having similar, conservatively related chemical properties such as hydrophobicity, hydrophilicity, electronegativity, small or bulky side chains, and the like. Residue positions which are not identical to the native peptide or protein sequence are thus replaced by amino acids having similar chemical properties, such as charge or polarity, where such changes are not likely to substantially effect the properties of the peptide or protein analog. These and other minor alterations will typically substantially maintain biological properties of the modified peptide or protein, including biological activity (e.g., binding to an adhesion molecule, or other ligand or receptor), immunoidentity (e.g., recognition by one or more monoclonal antibodies that recognize a native

peptide or protein), and other biological properties of the corresponding native peptide or protein.

As used herein, the term "conservative amino acid substitution" refers to the general interchangeability of amino acid residues having similar side chains. For example, a commonly interchangeable group of amino acids having aliphatic side chains is alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. Examples of conservative substitutions include the substitution of a non-polar (hydrophobic) residue such as isoleucine, valine, leucine or methionine for another. Likewise, the present invention contemplates the substitution of a polar (hydrophilic) residue such as between arginine and lysine, between glutamine and asparagine, and between threonine and serine. Additionally, the substitution of a basic residue such as lysine, arginine or histidine for another or the substitution of an acidic residue such as aspartic acid or glutamic acid for another is also contemplated. Exemplary conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine.

The term biologically active peptide or protein analog further includes modified forms of a native peptide or protein incorporating stereoisomers (e.g., D-amino acids) of the twenty conventional amino acids, or unnatural amino acids such as α,α-disubstituted amino acids, N-alkyl amino acids, lactic acid. These and other unconventional amino acids may also be substituted or inserted within native peptides and proteins useful within the invention. Examples of unconventional amino acids include: 4-hydroxyproline, γ-carboxyglutamate, ε-N,N,N-trimethyllysine, ε-N-acetyllysine, O-phosphoserine, N-acetylserine, N-formylmethionine, 3-methylhistidine, 5-hydroxylysine, ω-N-methylarginine, and other similar amino acids and imino acids (e.g., 4-hydroxyproline). In addition, biologically active peptide or protein analogs include single or multiple substitutions, deletions and/or additions of carbohydrate, lipid and/or proteinaceous moieties that occur naturally or artificially as structural components of the subject peptide or protein, or are bound to or otherwise associated with the peptide or protein.

To facilitate production and use of peptide and protein analogs within the invention, reference can be made to molecular phylogenetic studies that characterize conserved and divergent protein structural and functional elements between different members of a species, genus, family or other taxonomic group (e.g., between different human JAM, claudin, or occludin protein family members, allelic variants, and/or naturally occurring mutants, or between JAM, claudin, or occludin proteins found in different species, such as human, murine, rat and/or bovine JAM-1). In this regard, available studies will provide detailed assessments of structure-function relationships on a fine molecular level for modifying the majority of peptides and proteins disclosed herein to facilitate production and selection of operable peptide and protein analogs, including for membrane adhesive proteins, such as JAM, and other biologically active peptides and proteins disclosed herein for use within the invention. These studies include, for example, detailed sequence comparisons identifying conserved and divergent structural elements among, for example, multiple isoforms or species or allelic variants of a subject membrane adhesive peptide or protein. Each of these conserved and divergent structural elements facilitate practice of the invention by pointing to useful targets for modifying native peptides and proteins to confer desired structural and/or functional changes.

In this context, existing sequence alignments may be analyzed and conventional sequence alignment methods may be employed to yield sequence comparisons for analysis, for example to identify corresponding protein regions and amino acid positions between protein family members within a species, and between species variants of a protein of interest. These comparisons are useful to identify conserved and divergent structural elements of interest, the latter of which will often be useful for incorporation in a biologically active peptide or protein to yield a functional analog thereof. Typically, one or more amino acid residues marking a divergent structural element of interest in a different reference peptide sequence is incorporated within the functional peptide or protein analog. For example, a cDNA encoding a native JAM, occludin, or claudin peptide or protein may be recombinantly modified at one or more corresponding amino acid position(s) (i.e., corresponding positions that match or span a similar aligned sequence element according to accepted alignment methods to residues marking the structural element of interest in a heterologous reference peptide or protein sequence, such as an isoform, species or allelic variant, or synthetic mutant, of the subject JAM, occludin, or claudin peptide or

protein) to encode an amino acid deletion, substitution, or insertion that alters corresponding residue(s) in the native peptide or protein to generate an operable peptide or protein analog within the invention—having an analogous structural and/or functional element as the reference peptide or protein.

Within this rational design method for constructing biologically active peptide and protein analogs, the native or wild-type identity of residue(s) at amino acid positions corresponding to a structural element of interest in a heterologous reference peptide or protein may be altered to the same, or a conservatively related, residue identity as the corresponding amino acid residue(s) in the reference peptide or protein. However, it is often possible to alter native amino acid residues non-conservatively with respect to the corresponding reference protein residue(s). In particular, many non-conservative amino acid substitutions, particularly at divergent sites suggested to be more amenable to modification, may yield a moderate impairment or neutral effect, or even enhance a selected biological activity, compared to the function of a native peptide or protein.

Sequence alignment and comparisons to forecast useful peptide and protein analogs and mimetics will be further refined by analysis of crystalline structure (see, e.g., Löebermann et al., J. Molec. Biol. 177:531-556, 1984; Huber et al., Biochemistry 28:8951-8966, 1989; Stein et al., Nature 347:99-102, 1990; Wei et al., Structural Biology 1:251-255, 1994) of native biologically active proteins and peptides, coupled with computer modeling methods known in the art. These analyses allow detailed structure-function mapping to identify desired structural elements and modifications for incorporation into peptide and protein analogs and mimetics that will exhibit substantial activity comparable to that of the native peptide or protein for use within the methods and compositions of the invention.

Biologically active peptide and protein analogs of the invention typically show substantial sequence identity to a corresponding native peptide or protein sequence. The term "substantial sequence identity" means that the two subject amino acid sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap penalties, share at least 65 percent sequence identity, commonly 80 percent sequence identity, often at least 90-95 percent or greater sequence identity. "Percentage amino acid identity" refers to a comparison of the amino acid sequences of two peptides or proteins which, when optimally aligned, have approximately the

designated percentage of the same amino acids. Sequence comparisons are generally made to a reference sequence over a comparison window of at least 10 residue positions, frequently over a window of at least 15-20 amino acids, wherein the percentage of sequence identity is calculated by comparing a reference sequence to a second sequence, the latter of which may represent, for example, a peptide analog sequence that includes one or more deletions, substitutions or additions which total 20 percent, typically less than 5-10% of the reference sequence over the window of comparison. The reference sequence may be a subset of a larger sequence, for example, as a segment of a JAM, occludin, or claudin protein. Optimal alignment of sequences (e.g., alignment of human JAM-1 with human JAM-2 and/or JAM-3, or with another mammalian JAM protein) for aligning a comparison window may be conducted according to the local homology algorithm of Smith and Waterman (Adv. Appl. Math. 2:482, 1981), by the homology alignment algorithm of Needleman and Wunsch (J. Mol. Biol. 48:443, 1970), by the search for similarity method of Pearson and Lipman (Proc. Natl. Acad. Sci.USA 85:2444, 1988), or by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and/or TFASTA, e.g., as provided in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, WI).

By aligning a peptide or protein analog optimally with a corresponding native peptide or protein, and by using appropriate assays, e.g., adhesion protein or receptor binding assays, to determine a selected biological activity, one can readily identify operable peptide and protein analogs for use within the methods and compositions of the invention. Operable peptide and protein analogs are typically specifically immunoreactive with antibodies raised to the corresponding native peptide or protein. Likewise, nucleic acids encoding operable peptide and protein analogs will share substantial sequence identity as described above to a nucleic acid encoding the corresponding native peptide or protein, and will typically selectively hybridize to a partial or complete nucleic acid sequence encoding the corresponding native peptide or protein, or fragment thereof, under accepted, moderate or high stringency hybridization conditions (see, e.g., Sambrook et al., Molecular Cloning: A Laboratory Manual, 3rd Edition, Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y., 2001). The phrase "selectively hybridizing to" refers to a selective interaction between a nucleic acid probe that hybridizes, duplexes or binds preferentially to a particular target DNA or RNA sequence, for example when the target sequence is

present in a heterogenous preparation such as total cellular DNA or RNA. Generally, nucleic acid sequences encoding biologically active peptide and protein analogs, or fragments thereof, will hybridize to nucleic acid sequences encoding the corresponding native peptide or protein under stringent conditions (e.g., selected to be about 5°C lower than the thermal melting point (Tm) for the subject sequence at a defined ionic strength and pH, where the Tm is the temperature under defined ionic strength and pH at which 50% of the complementary or target sequence hybridizes to a perfectly matched probe). For discussions of nucleic acid probe design and annealing conditions, see, for example, Sambrook et al., Molecular Cloning: A Laboratory Manual, 3rd Edition, Vols. 1-3, Cold Spring Harbor Laboratory, 2001 or Current Protocols in Molecular Biology, F. Ausubel et al, ed., Greene Publishing and Wiley-Interscience, New York, 1987. Typically, stringent or selective conditions will be those in which the salt concentration is at least about 0.02 molar at pH 7 and the temperature is at least about 60°C. Less stringent selective hybridization conditions may also be chosen. As other factors may significantly affect the stringency of hybridization, including, among others, base composition and size of the complementary strands, the presence of organic solvents and the extent of base mismatching, the combination of parameters is more important than the specific measure of any one.

Within additional aspects of the invention, peptide mimetics are provided which comprise a peptide or non-peptide molecule that mimics the tertiary binding structure and activity of a selected native peptide or protein functional domain (e.g., binding motif or active site). These peptide mimetics include recombinantly or chemically modified peptides, as well as non-peptide agents such as small molecule drug mimetics, as further described below.

In one aspect, peptides (including polypeptides) useful within the invention are modified to produce peptide mimetics by replacement of one or more naturally occurring side chains of the 20 genetically encoded amino acids (or D amino acids) with other side chains, for instance with groups such as alkyl, lower alkyl, cyclic 4-, 5-, 6-, to 7-membered alkyl, amide, amide lower alkyl, amide di(lower alkyl), lower alkoxy, hydroxy, carboxy and the lower ester derivatives thereof, and with 4-, 5-, 6-, to 7-membered heterocyclics. For example, proline analogs can be made in which the ring size of the proline residue is changed from 5 members to 4, 6, or 7 members.

Cyclic groups can be saturated or unsaturated, and if unsaturated, can be aromatic or non-aromatic. Heterocyclic groups can contain one or more nitrogen, oxygen, and/or sulphur heteroatoms. Examples of such groups include the furazanyl, furyl, imidazolidinyl, imidazolyl, imidazolinyl, isothiazolyl, isoxazolyl, morpholinyl (e.g. morpholino), oxazolyl, piperazinyl (e.g. 1-piperazinyl), piperidyl (e.g. 1-piperidyl, piperidino), pyranyl, pyrazinyl, pyrazolidinyl, pyrazolinyl, pyrazolyl, pyridazinyl, pyridyl, pyrimidinyl, pyrrolidinyl (e.g. 1-pyrrolidinyl), pyrrolinyl, pyrrolyl, thiadiazolyl, thiazolyl, thienyl, thiomorpholinyl (e.g. thiomorpholino), and triazolyl. These heterocyclic groups can be substituted or unsubstituted. Where a group is substituted, the substituent can be alkyl, alkoxy, halogen, oxygen, or substituted or unsubstituted phenyl.

Peptides and proteins, as well as peptide and protein analogs and mimetics, can also be covalently bound to one or more of a variety of nonproteinaceous polymers, e.g., polyethylene glycol, polypropylene glycol, or polyoxyalkenes, in the manner set forth in U.S. Pat. No. 4,640,835; U.S. Pat. No. 4,496,689; U.S. Pat. No. 4,301,144; U.S. Pat. No. 4,670,417; U.S. Pat. No. 4,791,192; or U.S. Pat. No. 4,179,337.

Other peptide and protein analogs and mimetics within the invention include glycosylation variants, and covalent or aggregate conjugates with other chemical moieties. Covalent derivatives can be prepared by linkage of functionalities to groups which are found in amino acid side chains or at the N- or C- termini, by means which are well known in the art. These derivatives can include, without limitation, aliphatic esters or amides of the carboxyl terminus, or of residues containing carboxyl side chains, O-acyl derivatives of hydroxyl group-containing residues, and N-acyl derivatives of the amino terminal amino acid or amino-group containing residues, e.g., lysine or arginine. Acyl groups are selected from the group of alkyl-moieties including C3 to C18 normal alkyl, thereby forming alkanoyl aroyl species. Covalent attachment to carrier proteins, e.g., immunogenic moieties may also be employed.

In addition to these modifications, glycosylation alterations of biologically active peptides and proteins can be made, e.g., by modifying the glycosylation patterns of a peptide during its synthesis and processing, or in further processing steps. Particularly preferred means for accomplishing this are by exposing the peptide to glycosylating enzymes derived from cells that normally provide such processing, e.g., mammalian glycosylation enzymes. Deglycosylation enzymes can also be

successfully employed to yield useful modified peptides and proteins within the invention. Also embraced are versions of a native primary amino acid sequence which have other minor modifications, including phosphorylated amino acid residues, e.g., phosphotyrosine, phosphoserine, or phosphothreonine, or other moieties, including ribosyl groups or cross-linking reagents.

Peptidomimetics may also have amino acid residues that have been chemically modified by phosphorylation, sulfonation, biotinylation, or the addition or removal of other moieties, particularly those that have molecular shapes similar to phosphate groups. In some embodiments, the modifications will be useful labeling reagents, or serve as purification targets, e.g., affinity ligands.

A major group of peptidomimetics within the invention comprises covalent conjugates of native peptides or proteins, or fragments thereof, with other proteins or peptides. These derivatives can be synthesized in recombinant culture such as N- or C-terminal fusions or by the use of agents known in the art for their usefulness in cross-linking proteins through reactive side groups. Preferred peptide and protein derivatization sites for targeting by cross-linking agents are at free amino groups, carbohydrate moieties, and cysteine residues.

Fusion polypeptides between biologically active peptides or proteins and other homologous or heterologous peptides and proteins are also provided. Many growth factors and cytokines are homodimeric entities, and a repeat construct of these molecules or active fragments thereof will yield various advantages, including lessened susceptibility to proteolytic degradation. Repeat and other fusion constructs of membrane adhesive proteins, including JAM, occludin, and claudin, yield similar advantages within the methods and compositions of the invention. Various alternative multimeric constructs comprising peptides and proteins useful within the invention are thus provided. In certain embodiments, biologically active polypeptide fusions are provided as described in U.S. Patent No.s 6,018,026, 5,843,725, 6,291,646, 6,300,099, and 6,323,323, for example by linking one or more biologically active peptides or proteins of the invention with a heterologous, multimerizing polypeptide or protein, for example an immunoglobulin heavy chain constant region, or an immunoglobulin light chain constant region. The biologically active, multimerized polypeptide fusion thus constructed can be a hetero- or homo-multimer, e.g., a heterodimer or homodimer comprising one or more JAM, occludin, or claudin protein or peptide element(s), which may each comprise one or more distinct biologically

active peptides or proteins operable within the invention. Other heterologous polypeptides may be combined with the active peptide or protein to yield fusions that exhibit a combination of properties or activities of the derivative proteins. Other typical examples are fusions of a reporter polypeptide, e.g., CAT or luciferase, with a peptide or protein as described herein, to facilitate localization of the fused peptide or protein (see, e.g., Dull et al., U.S. Pat. No. 4,859,609,). Other fusion partners useful in this context include bacterial beta-galactosidase, trpE, Protein A, beta-lactamase, alpha amylase, alcohol dehydrogenase, and yeast alpha mating factor (see, e.g., Godowski et al., Science 241:812-816, 1988,).

The present invention also contemplates the use of biologically active peptides and proteins, including JAM, occludin and claudin peptides and proteins, modified by covalent or aggregative association with chemical moieties. These derivatives generally fall into the three classes: (1) salts, (2) side chain and terminal residue covalent modifications, and (3) adsorption complexes, for example with cell membranes. Such covalent or aggregative derivatives are useful for various purposes, for example to block homo- or heterotypic association between one or more JAM, occludin and claudin proteins, as immunogens, as reagents in immunoassays, or in purification methods such as for affinity purification of ligands or other binding ligands. For example, an active peptide or protein can be immobilized by covalent bonding to a solid support such as cyanogen bromide-activated Sepharose, by methods which are well known in the art, or adsorbed onto polyolefin surfaces, with or without glutaraldehyde cross-linking, for use in the assay or purification of antibodies that specifically bind the active peptide or protein. The active peptide or protein can also be labeled with a detectable group, for example radioiodinated by the chloramine T procedure, covalently bound to rare earth chelates, or conjugated to another fluorescent moiety for use in diagnostic assays, including assays involving intranasal administration of the labeled peptide or protein.

Those of skill in the art recognize that a variety of techniques are available for constructing peptide and protein mimetics with the same or similar desired biological activity as the corresponding native peptide or protein but with more favorable activity than the peptide or protein, for example improved characteristics of solubility, stability, and/or susceptibility to hydrolysis or proteolysis (see, e.g., Morgan and Gainor, Ann. Rep. Med. Chem. 24:243-252, 1989). Certain peptidomimetic compounds are based upon the amino acid sequence of the proteins and peptides

described herein for use within the invention, including sequences of JAM, occludin, and claudin proteins and peptides. Typically, peptidomimetic compounds are synthetic compounds having a three-dimensional structure (of at least part of the mimetic compound) that mimics, e.g., the primary, secondary, and/or tertiary structural, and/or electrochemical characteristics of a selected peptide or protein, or a structural domain, active site, or binding region (e.g., a homotypic or heterotypic binding site, catalytic active site or domain, receptor or ligand binding interface or domain, etc.) thereof. The peptide-mimetic structure or partial structure (also referred to as a peptidomimetic "motif" of a peptidomimetic compound) will share a desired biological activity with a native peptide or protein, e.g., activity to block homo- or heterotypic association between one or more JAM, occludin and claudin proteins, receptor binding and/or activation activities, immunogenic activity (such as binding to MHC molecules of one or multiple haplotypes and activating $CD8^+$ and/or $CD4^+$ T). Typically, the subject biologically activity of the mimetic compound is not substantially reduced in comparison to, and is often the same as or greater than, the activity of the native peptide on which the mimetic was modeled. In addition, peptidomimetic compounds can have other desired characteristics that enhance their therapeutic application, such as increased cell permeability, greater affinity and/or avidity, and prolonged biological half-life. The peptidomimetics of the invention will sometimes have a "backbone" that is partially or completely non-peptide, but with side groups identical to the side groups of the amino acid residues that occur in the peptide or protein on which the peptidomimetic is modeled. Several types of chemical bonds, e.g. ester, thioester, thioamide, retroamide, reduced carbonyl, dimethylene and ketomethylene bonds, are known in the art to be generally useful substitutes for peptide bonds in the construction of protease-resistant peptidomimetics.

The following describes methods for preparing peptide and protein mimetics modified at the N-terminal amino group, the C-terminal carboxyl group, and/or changing ore or more of the amido linkages in the peptide to a non-amido linkage. It being understood that two or more such modifications can be coupled in one peptide or protein mimetic structure (e.g., modification at the C-terminal carboxyl group and inclusion of a --CH_2 -carbamate linkage between two amino acids in the peptide. For N-terminal modifications, peptides typically are synthesized as the free acid but, as noted above, can be readily prepared as the amide or ester. One can also modify the

amino and/or carboxy terminus of peptide compounds to produce other compounds useful within the invention. Amino terminus modifications include methylating (i.e., --$NHCH_3$ or --$NH(CH_3)_2$), acetylating, adding a carbobenzoyl group, or blocking the amino terminus with any blocking group containing a carboxylate functionality defined by RCOO--, where R is selected from the group consisting of naphthyl, acridinyl, steroidyl, and similar groups. Carboxy terminus modifications include replacing the free acid with a carboxamide group or forming a cyclic lactam at the carboxy terminus to introduce structural constraints. Amino terminus modifications are as recited above and include alkylating, acetylating, adding a carbobenzoyl group, forming a succinimide group, etc. The N-terminal amino group can then be reacted as follows:

(a) to form an amide group of the formula RC(O)NH-- where R is as defined above by reaction with an acid halide [e.g., RC(O)Cl] or acid anhydride. Typically, the reaction can be conducted by contacting about equimolar or excess amounts (e.g., about 5 equivalents) of an acid halide to the peptide in an inert diluent (e.g., dichloromethane) preferably containing an excess (e.g., about 10 equivalents) of a tertiary amine, such as diisopropylethylamine, to scavenge the acid generated during reaction. Reaction conditions are otherwise conventional (e.g., room temperature for 30 minutes). Alkylation of the terminal amino to provide for a lower alkyl N-substitution followed by reaction with an acid halide as described above will provide for N-alkyl amide group of the formula RC(O)NR--;

(b) to form a succinimide group by reaction with succinic anhydride. As before, an approximately equimolar amount or an excess of succinic anhydride (e.g., about 5 equivalents) can be employed and the amino group is converted to the succinimide by methods well known in the art including the use of an excess (e.g., ten equivalents) of a tertiary amine such as diisopropylethylamine in a suitable inert solvent (e.g., dichloromethane) (see, for example, Wollenberg, et al., U.S. Pat. No. 4,612,132). It is understood that the succinic group can be substituted with, for example, C_2 -C_6 alkyl or --SR substituents that are prepared in a conventional manner to provide for substituted succinimide at the N-terminus of the peptide. Such alkyl substituents are prepared by reaction of a lower olefin (C_2 -C_6) with maleic anhydride in the manner described by Wollenberg, et al. (U.S. Pat. No. 4,612,132) and --SR substituents are prepared by reaction of RSH with maleic anhydride where R is as defined above;

(c) to form a benzyloxycarbonyl--NH-- or a substituted benzyloxycarbonyl--NH-- group by reaction with approximately an equivalent amount or an excess of CBZ-Cl (i.e., benzyloxycarbonyl chloride) or a substituted CBZ-Cl in a suitable inert diluent (e.g., dichloromethane) preferably containing a tertiary amine to scavenge the acid generated during the reaction;

(d) to form a sulfonamide group by reaction with an equivalent amount or an excess (e.g., 5 equivalents) of $R\text{-}S(O)_2Cl$ in a suitable inert diluent (dichloromethane) to convert the terminal amine into a sulfonamide where R is as defined above. Preferably, the inert diluent contains excess tertiary amine (e.g., ten equivalents) such as diisopropylethylamine, to scavenge the acid generated during reaction. Reaction conditions are otherwise conventional (e.g., room temperature for 30 minutes);

(e) to form a carbamate group by reaction with an equivalent amount or an excess (e.g., 5 equivalents) of R-OC(O)Cl or $R\text{-}OC(O)OC_6H_4$ -p-NO_2 in a suitable inert diluent (e.g., dichloromethane) to convert the terminal amine into a carbamate where R is as defined above. Preferably, the inert diluent contains an excess (e.g., about 10 equivalents) of a tertiary amine, such as diisopropylethylamine, to scavenge any acid generated during reaction. Reaction conditions are otherwise conventional (e.g., room temperature for 30 minutes);

(f) to form a urea group by reaction with an equivalent amount or an excess (e.g., 5 equivalents) of R--N=C=O in a suitable inert diluent (e.g., dichloromethane) to convert the terminal amine into a urea (i.e., RNHC(O)NH--) group where R is as defined above. Preferably, the inert diluent contains an excess (e.g., about 10 equivalents) of a tertiary amine, such as diisopropylethylamine. Reaction conditions are otherwise conventional (e.g., room temperature for about 30 minutes).

In preparing peptide mimetics wherein the C-terminal carboxyl group is replaced by an ester (i.e., --C(O)OR where R is as defined above), resins as used to prepare peptide acids are typically employed, and the side chain protected peptide is cleaved with base and the appropriate alcohol, e.g., methanol. Side chain protecting groups are then removed in the usual fashion by treatment with hydrogen fluoride to obtain the desired ester.

In preparing peptide mimetics wherein the C-terminal carboxyl group is replaced by the amide --$C(O)NR_3R_4$, a benzhydrylamine resin is used as the solid support for peptide synthesis. Upon completion of the synthesis, hydrogen fluoride treatment to release the peptide from the support results directly in the free peptide

amide (i.e., the C-terminus is --C(O)NH_2). Alternatively, use of the chloromethylated resin during peptide synthesis coupled with reaction with ammonia to cleave the side chain protected peptide from the support yields the free peptide amide and reaction with an alkylamine or a dialkylamine yields a side chain protected alkylamide or dialkylamide (i.e., the C-terminus is --C(O)NRR_1 where R and R_1 are as defined above). Side chain protection is then removed in the usual fashion by treatment with hydrogen fluoride to give the free amides, alkylamides, or dialkylamides.

In another alternative embodiments of the invention, the C-terminal carboxyl group or a C-terminal ester of a biologically active peptide can be induced to cyclize by internal displacement of the --OH or the ester (--OR) of the carboxyl group or ester respectively with the N-terminal amino group to form a cyclic peptide. For example, after synthesis and cleavage to give the peptide acid, the free acid is converted to an activated ester by an appropriate carboxyl group activator such as dicyclohexylcarbodiimide (DCC) in solution, for example, in methylene chloride (CH_2Cl_2), dimethyl formamide (DMF) mixtures. The cyclic peptide is then formed by internal displacement of the activated ester with the N-terminal amine. Internal cyclization as opposed to polymerization can be enhanced by use of very dilute solutions. Such methods are well known in the art.

One can cyclize active peptides for use within the invention, or incorporate a desamino or descarboxy residue at the termini of the peptide, so that there is no terminal amino or carboxyl group, to decrease susceptibility to proteases, or to restrict the conformation of the peptide. C-terminal functional groups among peptide analogs and mimetics of the present invention include amide, amide lower alkyl, amide di(lower alkyl), lower alkoxy, hydroxy, and carboxy, and the lower ester derivatives thereof, and the pharmaceutically acceptable salts thereof.

Other methods for making peptide and protein derivatives and mimetics for use within the methods and compositions of the invention are described in Hrúby et al. (Biochem J. 268(2):249-262, 1990). According to these methods, biologically active peptides and proteins serve as structural models for non-peptide mimetic compounds having similar biological activity as the native peptide or protein. Those of skill in the art recognize that a variety of techniques are available for constructing compounds with the same or similar desired biological activity as the lead peptide or protein compound, or that have more favorable activity than the lead with respect a desired property such as solubility, stability, and susceptibility to hydrolysis and

proteolysis (see, e.g., Morgan and Gainor, Ann. Rep. Med. Chem. 24:243-252, 1989). These techniques include, for example, replacing a peptide backbone with a backbone composed of phosphonates, amidates, carbamates, sulfonamides, secondary amines, and/or N-methylamino acids.

Peptide and protein mimetics wherein one or more of the peptidyl linkages [--C(O)NH--] have been replaced by such linkages as a --CH_2 -carbamate linkage, a phosphonate linkage, a --CH_2 -sulfonamide linkage, a urea linkage, a secondary amine (--CH_2NH--) linkage, and an alkylated peptidyl linkage [--C(O)NR_6 -- where R_6 is lower alkyl] are prepared, for example, during conventional peptide synthesis by merely substituting a suitably protected amino acid analogue for the amino acid reagent at the appropriate point during synthesis. Suitable reagents include, for example, amino acid analogues wherein the carboxyl group of the amino acid has been replaced with a moiety suitable for forming one of the above linkages. For example, if one desires to replace a --C(O)NR-- linkage in the peptide with a --CH_2 -carbamate linkage (--$CH_2OC(O)NR$--), then the carboxyl (--COOH) group of a suitably protected amino acid is first reduced to the --CH_2OH group which is then converted by conventional methods to a --OC(O)Cl functionality or a para-nitrocarbonate --OC(O)O-C_6H_4-p-NO_2 functionality. Reaction of either of such functional groups with the free amine or an alkylated amine on the N-terminus of the partially fabricated peptide found on the solid support leads to the formation of a --$CH_2OC(O)NR$-- linkage. For a more detailed description of the formation of such --CH_2 -carbamate linkages, see, e.g., Cho et al. (Science 261:1303-1305, 1993).

Replacement of an amido linkage in an active peptide with a --CH_2 -sulfonamide linkage can be achieved by reducing the carboxyl (--COOH) group of a suitably protected amino acid to the --CH_2OH group, and the hydroxyl group is then converted to a suitable leaving group such as a tosyl group by conventional methods. Reaction of the derivative with, for example, thioacetic acid followed by hydrolysis and oxidative chlorination will provide for the --CH_2--$S(O)_2Cl$ functional group which replaces the carboxyl group of the otherwise suitably protected amino acid. Use of this suitably protected amino acid analogue in peptide synthesis provides for inclusion of an --$CH_2S(O)_2NR$-- linkage that replaces the amido linkage in the peptide thereby providing a peptide mimetic. For a more complete description on the conversion of the carboxyl group of the amino acid to a --$CH_2S(O)_2Cl$ group, see, e.g., Weinstein and Boris (Chemistry & Biochemistry of Amino Acids, Peptides and Proteins, Vol. 7,

pp. 267-357, Marcel Dekker, Inc., New York, 1983). Replacement of an amido linkage in an active peptide with a urea linkage can be achieved, for example, in the manner set forth in U.S. Patent Application Ser. No. 08/147,805.

Secondary amine linkages wherein a --CH_2NH-- linkage replaces the amido linkage in the peptide can be prepared by employing, for example, a suitably protected dipeptide analogue wherein the carbonyl bond of the amido linkage has been reduced to a CH_2 group by conventional methods. For example, in the case of diglycine, reduction of the amide to the amine will yield after deprotection $H_2NCH_2CH_2NHCH_2$ COOH that is then used in N-protected form in the next coupling reaction. The preparation of such analogues by reduction of the carbonyl group of the amido linkage in the dipeptide is well known in the art.

The biologically active peptide and protein agents of the present invention may exist in a monomeric form with no disulfide bond formed with the thiol groups of cysteine residue(s) that may be present in the subject peptide or protein. Alternatively, an intermolecular disulfide bond between thiol groups of cysteines on two or more peptides or proteins can be produced to yield a multimeric (e.g., dimeric, tetrameric or higher oligomeric) compound. Certain of such peptides and proteins can be cyclized or dimerized via displacement of the leaving group by the sulfur of a cysteine or homocysteine residue (see, e.g., Barker et al., J. Med. Chem. 35:2040-2048, 1992; and Or et al., J. Org. Chem. 56:3146-3149, 1991,). Thus, one or more native cysteine residues may be substituted with a homocysteine. Intramolecular or intermolecular disulfide derivatives of active peptides and proteins provide analogs in which one of the sulfurs has been replaced by a CH_2 group or other isostere for sulfur. These analogs can be made via an intramolecular or intermolecular displacement, using methods known in the art.

All of the naturally occurring, recombinant, and synthetic peptides and proteins, and the peptide and protein analogs and mimetics, identified as useful agents within the invention can be used for screening (e.g., in kits and/or screening assay methods) to identify additional compounds, including other peptides, proteins, analogs and mimetics, that will function within the methods and compositions of the invention, including as inhibitors of homotypic and heterotypic binding between membrane adhesive proteins to enhance epithelial permeability. Several methods of automating assays have been developed in recent years so as to permit screening of tens of thousands of compounds in a short period (see, e.g., Fodor et al., Science

251:767-773, 1991, and U.S. Patent Nos. 5,677,195; 5,885,837; 5,902,723; 6,027,880; 6,040,193; and 6,124,102, issued to Fodor et al.). Large combinatorial libraries of compounds can be constructed by encoded synthetic libraries (ESL) described in, e.g., WO 95/12608, WO 93/06121, WO 94/08051, WO 95/35503, and WO 95/30642. Peptide libraries can also be generated by phage display methods (see, e.g., Devlin, W0 91/18980).

One method of screening for new biologically active agents for use within the invention (e.g., small molecule drug peptide mimetics) utilizes eukaryotic or prokaryotic host cells which are stably transformed with recombinant DNA molecules expressing an active peptide or protein, e.g., a JAM, occludin, or claudin peptide or protein. Such cells, either in viable or fixed form, can be used for standard assays, e.g., ligand/receptor binding assays (see, e.g., Parce et al., Science 246:243-247, 1989; and Owicki et al., Proc. Natl. Acad. Sci. USA 87:4007-4011, 1990). Competitive assays are particularly useful, for example assays where the cells are contacted and incubated with a labeled receptor or antibody having known binding affinity to the peptide ligand, and a test compound or sample whose binding affinity is being measured. The bound and free labeled binding components are then separated to assess the degree of ligand binding. The amount of test compound bound is inversely proportional to the amount of labeled receptor binding to the known source. Any one of numerous techniques can be used to separate bound from free ligand to assess the degree of ligand binding. This separation step can involve a conventional procedure such as adhesion to filters followed by washing, adhesion to plastic followed by washing, or centrifugation of the cell membranes.

Another technique for drug screening within the invention involves an approach which provides high throughput screening for compounds having suitable binding affinity to a target molecule, e.g., a JAM, occludin, or claudin protein, and is described in detail in Geysen, European Patent Application 84/03564, published on Sep. 13, 1984. First, large numbers of different test compounds, e.g., small peptides, are synthesized on a solid substrate, e.g., plastic pins or some other appropriate surface, (see, e.g., Fodor et al., Science 251:767-773, 1991, and U.S. Patent Nos. 5,677,195; 5,885,837; 5,902,723; 6,027,880; 6,040,193; and 6,124,102, issued to Fodor et al.). Then all of the pins are reacted with a solubilized peptide agent of the invention, and washed. The next step involves detecting bound peptide.

Rational drug design may also be based upon structural studies of the molecular shapes of biologically active peptides and proteins determined to operate within the methods of the invention. Various methods are available and well known in the art for characterizing, mapping, translating, and reproducing structural features of peptides and proteins to guide the production and selection of new peptide mimetics, including for example x-ray crystallography and 2 dimensional NMR techniques. These and other methods, for example, will allow reasoned prediction of which amino acid residues present in a selected peptide or protein form molecular contact regions necessary for specificity and activity (see, e.g., Blundell and Johnson, Protein Crystallography, Academic Press, N.Y., 1976).

Operable analogs and mimetics of JAM, occludin and claudin and of other biologically active agents disclosed herein retain partial, complete or enhanced activity compared to a native peptides, protein or unmodified compound. For example analogs or mimetics of JAM will exhibit partial or complete activity for homotypic or heterotypic binding exhibited by the corresponding native or wild-type JAM protein or peptide. In this regard, operable analogs and mimetics for use within the invention will retain at least 50%, often 75%, and up to 95-100% or greater levels of one or more selected activities as compared to the same activity observed for a selected native peptide or protein or unmodified compound. These biological properties of altered peptides or non-peptide mimetics can be determined according to any suitable assay disclosed or incorporated herein, for example by determining the ability of a JAM, occludin, or claudin analog or mimetic to block homotypic or heterotypic binding of the corresponding native protein and/or to increase permeability of mucosal epithelial cells *in vivo* or *in vitro*.

In accordance with the description herein, the compounds of the invention are useful *in vitro* as unique tools for analyzing the nature and function of JAM, occludin and claudin proteins, and will therefore also serve as leads in various programs for designing additional peptide and non-peptide (e.g., small molecule drug) agents for enhancing mucosal epithelial permeability and facilitating mucosal drug delivery.

In addition, the JAM, occludin and claudin peptides, proteins, analogs and mimetics disclosed herein are useful as immunogens, or components of immunogens, for generating antibodies and related agents that will be useful, for example, to block homotypic or heterotypic binding between the corresponding native protein and/or effectuate permeabilization of mucosal epithelial cells. The peptides will be

administered as immunogens, typically in the form of a conjugate (e.g., a multimeric peptide, or a peptide/carrier or peptide/hapten conjugate), to generate antibodies that bind the immunizing peptide(s) or peptide conjugate(s) with high affinity or avidity, but do not similarly recognize unrelated peptides.

Thus, the invention also provides diagnostic and therapeutic antibodies, including monoclonal antibodies, directed against a JAM, occludin or claudin peptide or protein, including antibodies against specific portions or domains (e.g., a homotypic binding interface) of a JAM, occludin or claudin protein. The antibodies specifically recognize functional portions of the JAM, occludin or claudin protein, and are therefore useful for blocking interactions between these proteins, or permeabilizing mucosal epithelial target cells when administered *in vivo*. These immunotherapeutic reagents may include humanized antibodies, and can be combined for therapeutic use with additional active or inert ingredients as disclosed herein, e.g., in conventional pharmaceutically acceptable carriers or diluents, e.g., immunogenic adjuvants, and optionally with adjunctive or combinatorially active agents such as antiretroviral drugs. Methods for generating functional antibodies, including humanized antibodies, antibody fragments, and other related agents are well known in the art (see, e.g., Harlow & Lane, <u>Antibodies, A Laboratory Manual</u>, CSHP, NY, 1988; Queen et al., <u>Proc. Natl. Acad. Sci. USA</u> <u>86</u>:10029-10033, 1989 and WO 90/07861. Human antibodies can be obtained using phage-display methods (see, e.g., Dower et al., WO 91/17271; McCafferty et al., WO 92/01047,). Similarly, methods for producing active antibody fragments are well known, including methods for generating separate heavy chains, light chains Fab, Fab' F(ab')2, Fv, and single chain antibodies. Fragments can be produced by enzymic or chemical separation of intact immunoglobulins using standard methods, such as those described in Harlow and Lane, supra. Fab fragments may be obtained from F(ab')2 fragments by limited reduction, or from whole antibody by digestion with papain in the presence of reducing agents. Fragments can also be produced by recombinant DNA techniques. Segments of nucleic acids encoding selected fragments are produced by digestion of full-length coding sequences with restriction enzymes, or by *de novo* synthesis. Often fragments are expressed in the form of phage-coat fusion proteins. This manner of expression is advantageous for affinity-sharpening of antibodies.

The anti-JAM, occludin and claudin antibodies of the invention can also generally be used in drug screening compositions and procedures, as noted above, to

identify additional compounds having activity for interfering or blocking binding interactions of a JAM, occludin or claudin protein, and/or inducing increased permeability in mucosal epithelial cells. Various screening methods and formats for this purpose are available and well known in the art as discussed above. In such assays, the peptide and antibody compounds of the invention can be used without modification or can be modified in a variety of ways; for example, by labeling, such as covalently or non-covalently joining a moiety which directly or indirectly provides a detectable signal. Possibilities for direct labeling include label groups such as: radiolabels, enzymes such as peroxidase and alkaline phosphatase (see, e.g., U.S. Pat. No. 3,645,090; and U.S. Pat. No. 3,940,475), and fluorescent labels. Possibilities for indirect labeling include biotinylation of one constituent followed by binding to avidin coupled to one of the above label groups. The compounds may also include spacers or linkers in cases where the compounds are to be attached to a solid support.

The peptides and antibodies and other compounds of the present invention can also be utilized as commercial reagents for various medical research and diagnostic uses. Such uses include but are not limited to: (1) use as a calibration standard for quantifying the activities of agonists and antagonists of JAM, occludin and claudin peptides and proteins in a variety of functional assays; (2) use in structural analysis of JAM, occludin and claudin peptides and proteins; and (3) use to investigate the mechanism of action of JAM, occludin and claudin peptides and proteins.

A variety of additives, diluents, bases and delivery vehicles are provided within the invention that effectively control water content to enhance protein stability. These reagents and carrier materials effective as anti-aggregation agents in this sense include, for example, polymers of various functionalities, such as polyethylene glycol, dextran, diethylaminoethyl dextran, and carboxymethyl cellulose, which significantly increase the stability and reduce the solid-phase aggregation of peptides and proteins admixed therewith or linked thereto. In some instances, the activity or physical stability of proteins can also be enhanced by various additives to aqueous solutions of the peptide or protein drugs. For example, additives, such as polyols (including sugars), amino acids, proteins such as collagen and gelatin, and various salts may be used.

Certain additives, in particular sugars and other polyols, also impart significant physical stability to dry, e.g., lyophilized proteins. These additives can also be used within the invention to protect the proteins against aggregation not only during lyophilization but also during storage in the dry state. For example sucrose and Ficoll 70 (a polymer with sucrose units) exhibit significant protection against peptide or protein aggregation during solid-phase incubation under various conditions. These additives may also enhance the stability of solid proteins embedded within polymer matrices.

Yet additional additives, for example sucrose, stabilize proteins against solid-state aggregation in humid atmospheres at elevated temperatures, as may occur in certain sustained-release formulations of the invention. Proteins such as gelatin and collagen also serve as stabilizing or bulking agents to reduce denaturation and aggregation of unstable proteins in this context. These additives can be incorporated into polymeric melt processes and compositions within the invention. For example, polypeptide microparticles can be prepared by simply lyophilizing or spray drying a solution containing various stabilizing additives described above. Sustained release of unaggregated peptides and proteins can thereby be obtained over an extended period of time.

Various additional preparative components and methods, as well as specific formulation additives, are provided herein which yield formulations for mucosal delivery of aggregation-prone peptides and proteins, wherein the peptide or protein is stabilized in a substantially pure, unaggregated form. A range of components and additives are contemplated for use within these methods and formulations. Exemplary of these anti-aggregation agents are linked dimers of cyclodextrins (CDs), which selectively bind hydrophobic side chains of polypeptides (see, e.g., Breslow, et al., J. Am. Chem. Soc. 120:3536-3537; Maletic, et al., Angew. Chem. Int. Ed. Engl. 35:1490-1492). These CD dimers have been found to bind to hydrophobic patches of proteins in a manner that significantly inhibits aggregation (Leung et al., Proc. Nat.l Acad. Sci. USA 97:5050-5053, 2000). This inhibition is selective with respect to both the CD dimer and the protein involved. Such selective inhibition of protein aggregation provides additional advantages within the intranasal delivery methods and compositions of the invention. Additional agents for use in this context include CD trimers and tetramers with varying geometries controlled by the linkers that specifically block aggregation of peptides and proteins (Breslow et al., J. Am. Chem.

Soc. 118:11678-11681, 1996; Breslow et al., PNAS USA 94:11156-11158, 1997; Breslow et al., Tetrahedron Lett. 2887-2890, 1998).

Yet additional anti-aggregation agents and methods for incorporation within the invention involve the use of peptides and peptide mimetics to selectively block protein-protein interactions. In one aspect, the specific binding of hydrophobic side chains reported for CD multimers is extended to proteins via the use of peptides and peptide mimetics that similarly block protein aggregation. A wide range of suitable methods and anti-aggregation agents are available for incorporation within the compositions and procedures of the invention (Zutshi et al., Curr. Opin. Chem. Biol. 2:62-66, 1998; Daugherty et al., J. Am. Chem. Soc. 121:4325-4333, 1999: Zutshi et al., J. Am. Chem. Soc. 119:4841-4845, 1997; Ghosh et al, Chem. Biol. 5:439-445, 1997; Hamuro et al., Angew. Chem. Int. Ed. Engl. 36:2680-2683, 1997; Alberg et al., Science 262:248-250, 1993; Tauton et al., J. Am. Chem. Soc. 118:10412-10422, 1996; Park et al., J. Am. Chem. Soc. 121:8-13, 1999; Prasanna et al., Biochemistry 37:6883-6893, 1998; Tiley et al., J. Am. Chem. Soc. 119:7589-7590, 1997; Judice et al., PNAS, USA 94:13426-13430, 1997; Fan et al., J. Am. Chem. Soc. 120:8893-8894, 1998; Gamboni et al., Biochemistry 37:12189-12194, 1998). Briefly, these methods involve rational design and selection of peptides and mimetics that effectively block interactions between selected biologically active peptides or proteins, whereby the selected peptides and mimetics significantly reduce aggregation of the active peptides or proteins in a mucosal formulation. Anti-aggregation peptides and mimetics thus identified are coordinately administered with, or admixed or conjugated in a combinatorial formulation with, a biologically active peptide or protein to effectively inhibit aggregation of the active peptide or protein in a manner that significantly enhances absorption and/or bioavailability of the active peptide or protein.

Other techniques in peptide and protein engineering disclosed herein will further reduce the extent of protein aggregation and instability in mucosal delivery methods and formulations of the invention. One example of a useful method for peptide or protein modification in this context is PEGylation. The stability and aggregation problems of polypeptide drugs can be significantly improved by covalently conjugating water-soluble polymers such as PEG with the polypeptide. Another example is modification of a peptide or protein amino acid sequence in terms of the identity or location of one or more residues, e.g., by terminal or internal

addition, deletion or substitution (e.g., deletion of cysteine residues or replacement by alanine or serine) to reduce aggregation potential. The improvements in terms of stability and aggregation potential that are achieved by these methods enables effective mucosal delivery of a therapeutically effective polypeptide or protein composition within the methods of the invention.

CHARGE MODIFYING AND PH CONTROL AGENTS AND METHODS

To improve the transport characteristics of biologically active agents (e.g., macromolecular drugs, peptides or proteins) for enhanced delivery across hydrophobic mucosal membrane barriers, the invention also provides techniques and reagents for charge modification of selected biologically active agents or delivery-enhancing agents described herein. In this regard, the relative permeabilities of macromolecules is generally be related to their partition coefficients. The degree of ionization of molecules, which is dependent on the pK_a of the molecule and the pH at the mucosal membrane surface, also affects permeability of the molecules. Permeation and partitioning of biologically active agents and permeabilizing agents, including JAM, occludin, and claudin peptides and analogs of the invention, for mucosal delivery may be facilitated by charge alteration or charge spreading of the active agent or permeabilizing agent, which is achieved, for example, by alteration of charged functional groups, by modifying the pH of the delivery vehicle or solution in which the active agent is delivered, or by coordinate administration of a charge- or pH-altering reagent with the active agent.

A model compound for evaluating charge- and pH-modification methods for use within the mucosal delivery formulations and methods of the inventions is nicotine. The charge status of this model therapeutic as a function of pH has been investigated at various delivery sites of skin and absorptive mucosae (*see, e.g.*, Nair et al., J. Pharm. Sci. 86:257-262, 1997).

Consistent with these general teachings, mucosal delivery of charged macromolecular species, including JAM, occludin, and claudin peptides and other biologically active peptides and proteins, within the methods and compositions of the invention is substantially improved when the active agent is delivered to the mucosal surface in a substantially un-ionized, or neutral, electrical charge state.

Calculation of the isoelectric points of JAM, occludin, and claudin peptides and other biologically active peptides, proteins, and peptide analogs and mimetics is readily undertaken to guide the selection of pH and other values for mucosal formulations within the invention, which optionally deliver charged macromolecules in a substantially un-ionized state to the mucosal surface or, alternatively, following mucosal delivery at a target site of drug action. The p*I* of an amphoteric molecule is defined as the pH at which the net charge is zero. The variation of net charge with pH is of importance in charge-dependent separation methods like electrophoresis, isoelectric focusing, chromatofocusing and ion-exchange chromatography. Thus, methods for estimating isoelectric points (p*I*) for native peptides and proteins are well known and readily implemented within the methods and compositions of the invention (see, e.g., Cameselle, et al., Biochem. Educ. 14:131-136, 1986; Skoog, et al., Trends Anal. Chem. 5:82-83, 1986; Sillero et al., Anal. Biochem. 179:319-25, 1989; Englund, et al., Biochim. Biophys. Acta, 1065:185-194, 1991; Bjellquist et al., Electrophoresis. 14:1023-1031, 1993; Mosher et al., J. Chromatogr. 638:155-164, 1993; Bjellqvist et al., Electrophoresis 15:529-539, 1994; Watts, et al., Electrophoresis 16:22-27, 1995).

Certain JAM, occludin, and claudin peptides and other biologically active peptide and protein components of mucosal formulations for use within the invention will be charge modified to yield an increase in the positive charge density of the peptide or protein. These modifications extend also to cationization of peptide and protein conjugates, carriers and other delivery forms disclosed herein. Cationization offers a convenient means of altering the biodistribution and transport properties of proteins and macromolecules within the invention. Cationization is undertaken in a manner that substantially preserves the biological activity of the active agent and limits potentially adverse side effects, including tissue damage and toxicity. In many cases, cationized molecules have higher organ uptake and penetration compared with non-cationized forms (see, e.g., Ekrami et al., Journal of Pharmaceutical Sciences 84:456-461, 1995; Bergman et al., Clin. Sci. 67:35-43, 1984; Triguero et al., J. Pharm. Exp. Ther. 258:186-192, 1991). In some cases, cationized proteins can penetrate physiological barriers considered impenetrable by the native proteins. For example, cationized albumin (Pardridge et al., J. Pharm. Exp. Ther. 255:893-899, 1991,) and cationized IgG (Triguero et al., Proc. Nat. Acad. Sci. USA, 86:4761-4765, 1989,) have been demonstrated to bind to the brain capillary endothelium *in vitro* and cross

the blood-brain barrier *in vivo* to a much greater extent than native albumin and native IgG. Cationized proteins are also generally taken up by the lungs to a greater extent than native proteins (Bergman et al., Clin. Sci. 67:35-43, 1984; Triguero et al., J. Pharm. Exp. Ther. 258:186-192, 1991; Pardridge et al., J. Pharm. Exp. Ther. 251:821-826, 1989). At the tissue level, it has been demonstrated that cationized ferritin (CF) binds to and is transcytosed across the pulmonary endothelium (Pietra et al., Lab Invest. 49:54-61, 1983; Pietra et al., Lab Invest. 59:683-691, 1988) in isolated, perfused rat lungs, whereas native ferritin does not bind to the pulmonary endothelium and is only transcytosed across this barrier to a small degree. Bergman et al. (Clin. Sci. 67:35-43, 1984) demonstrated that by increasing the level of cationization and the charge density of human serum albumin (as measured by the change in the pI value of native albumin), the uptake of cationized albumins by the lungs following iv administration in rats can be increased. Pardridge et al. have also demonstrated that cationized IgG and physiologically cationic histone (Pardridge et al., J. Pharm. Exp. Ther. 251:821-826, 1989) have higher uptakes in the lungs compared with native IgG and bovine albumin, respectively. However, some studies have failed to demonstrate higher lung uptake for cationized proteins compared with native proteins. For instance, Pardridge et al (Pardridge et al., J. Pharm. Exp. Ther. 255:893-899, 1991,) and Takakura et al.(Takakura et al., Pharm. Res. 7:339-346, 1990) report lower lung uptake for cationized albumin compared with native albumin following iv biodistribution studies in animals.

DEGRADATIVE ENZYME INHIBITORY AGENTS AND METHODS

A major drawback to effective mucosal delivery of biologically active agents, including JAM, occludin, and claudin peptides, is that they may be subject to degradation by mucosal enzymes. The oral route of administration of therapeutic compounds is particularly problematic, because in addition to proteolysis in the stomach, the high acidity of the stomach destroys many active and inactive components of mucosal delivery formulations before they reach an intended target site of drug action. Further impairment of activity occurs by the action of gastric and pancreatic enzymes, and exo and endopeptidases in the intestinal brush border

membrane, and by metabolism in the intestinal mucosa where a penetration barrier substantially blocks passage of the active agent across the mucosa.

In addition to their susceptibility to enzymatic degradation, many therapeutic compounds, particularly relatively low molecular weight proteins, and peptides, introduced into the circulation, are cleared quickly from mammalian subjects by the kidneys. This problem may be partially overcome by administering large amounts of the therapeutic compound through repeated administration. However, higher doses of therapeutic formulations containing protein or peptide components can elicit antibodies that can bind and inactivate the protein and/or facilitate the clearance of the protein from the subject's body. Repeated administration of the formulation containing the therapeutic protein or peptide is essentially ineffective and can be dangerous as it can elicit an allergic or autoimmune response.

The problem of metabolic lability of therapeutic peptides, proteins and other compounds may be addressed in part through rational drug design. However, medicinal chemists have had less success in manipulating the structures of peptides and proteins to achieve high cell membrane permeability while still retaining pharmacological activity. Unfortunately, many of the structural features of peptides and proteins (e.g., free N-terminal amino and C-terminal carboxyl groups, and side chain carboxyl (e.g., Asp, Glu), amino (e.g., Lys, Arg) and hydroxyl (e.g. Ser, Thr, Tyr) groups) that bestow upon the molecule affinity and specificity for its pharmacological binding partner also bestow upon the molecule undesirable physicochemical properties (e.g., charge, hydrogen bonding potential) which limit their cell membrane permeability. Therefore, alternative strategies need to be considered for intranasal formulation and delivery of peptide and protein therapeutics.

Attempts to overcome the so-called enzymatic barrier to drug delivery include the use of liposomes (Takeuchi et al., Pharm. Res. 13:896-901, 1996) and nanoparticles (Mathiowitz et al., Nature. 386:410-4, 1997) that reportedly provide protection for incorporated insulin towards an enzymatic attack and the development of delivery systems targeting to the colon, where the enzymatic activity is comparatively low (Rubenstein et al., J. Control Rel. 46:59-73, 1997). In addition, co-administration of protease inhibitors has been reported in various studies to improve the oral bioavailability of insulin (Fujii et al, J. Pharm Pharmacol. 37:545-9, 1985; Yamamoto et al., Pharm Res. 11:1496-600, 1994; Moroshita et al., Int. J. Pharm. 78:9-16, 1992).

More recent research efforts in the area of protease inhibition for enhanced delivery of biotherapeutic compounds, including peptide and protein therapeutics, has focused on covalent immobilization of enzyme inhibitors on mucoadhesive polymers used as drug carrier matrices (see, e.g., Bernkop-Schnurch et al., Drug Dev. Ind. Pharm. 23:733-40, 1997; Bernkop-Schnurch et al., J. Control. Rel. 47:113-21, 1997; Bernkop-Schnurch et al., J. Drug Targ. 7:55-63, 1999). In conjunction with these teachings, the invention provides in more detailed aspects an enzyme inhibitor formulated with a common carrier or vehicle for mucosal delivery of JAM, occludin, and claudin peptides and other biologically active peptides, analogs and mimetics, optionally to be administered coordinately one or more additional biologically active or delivery-enhancing agents. Optionally, the enzyme inhibitor is covalently linked to the carrier or vehicle. In certain embodiments, the carrier or vehicle is a biodegradable polymer, for example, a bioadhesive polymer. Thus, for example, a protease inhibitor, such as Bowman-Birk inhibitor (BBI), displaying an inhibitory effect towards trypsin and ά-chymotrypsin (Birk Y. Int. J. Pept. Protein Res. 25:113-31, 1985), or elastatinal, an elastase-specific inhibitor of low molecular size, may be covalently linked to a mucoadhesive polymer as described herein. The resulting polymer-inhibitor conjugate exhibits substantial utility as a mucosal delivery vehicle for peptides and other biologically active agents formulated or delivered alone or in combination with other biologically active agents or additional delivery-enhancing agents.

Exemplary mucoadhesive polymer-enzyme inhibitor complexes that are useful within the mucosal delivery formulations and methods of the invention include, but are not limited to: Carboxymethylcellulose-pepstatin (with anti-pepsin activity); Poly(acrylic acid)-Bowman-Birk inhibitor (anti-chymotrypsin); Poly(acrylic acid)-chymostatin (anti-chymotrypsin); Poly(acrylic acid)-elastatinal (anti-elastase); Carboxymethylcellulose-elastatinal (anti-elastase); Polycarbophil—elastatinal (anti-elastase); Chitosan—antipain (anti-trypsin); Poly(acrylic acid)—bacitracin (anti-aminopeptidase N); Chitosan—EDTA (anti-aminopeptidase N, anti-carboxypeptidase A); Chitosan—EDTA—antipain (anti-trypsin, anti-chymotrypsin, anti-elastase) (see, e.g., Bernkop-Schnürch, J. Control. Rel. 52:1-16, 1998). As described in further detail below, certain embodiments of the invention will optionally incorporate a novel chitosan derivative or chemically modified form of chitosan. One such novel

derivative for use within the invention is denoted as a β-[1→4]-2-guanidino-2-deoxy-D-glucose polymer (poly-GuD).

In recent years the use of enzyme inhibitors to overcome the enzymatic barrier to perorally administered therapeutic peptides and proteins has gained considerable interest (for a detailed review, see, Bernkop-Schnürch, A. J. Control. Rel. 52:1—16, 1998,). However, especially for peptide and protein drugs that are used in long-term therapy, the co-administration of enzyme inhibitors has remained questionable because of side effects caused by these agents. Several side effects, such as systemic intoxications, a disturbed digestion of nutritive proteins, and hypertrophy as well as hyperplasia of the pancreas based on a feedback regulation, may accompany enzyme inhibitor co-administration by oral delivery methods. Even if systemic toxic side effects and an intestinal mucosal damage can be excluded, enzyme inhibitors of pancreatic proteases still have a toxic potential caused by the inhibition of these digestive enzymes themselves. Besides a disturbed digestion of nutritive proteins, an inhibitor-induced stimulation of protease secretion caused by a feed-back regulation may be expected [Reseland et al., Hum. Clin. Nutr. 126:634-642, (1996)]. Numerous studies have investigated this feed-back regulation with inhibitors, such as Bowman-Birk inhibitor, soybean trypsin inhibitor (Kunitz trypsin inhibitor) and camostat, in rats and mice. They demonstrate that this feed-back regulation rapidly leads to both hypertrophy and hyperplasia of the pancreas. Moreover, a prolonged oral administration of the Bowman-Birk inhibitor and soybean trypsin inhibitor leads to the development of numerous neoplastic foci, frequently progressing to invasive carcinoma [Otsuki et al., Pancreas 2:164-169, 1987; Melmed et al., Biochim. Biophys. Acta 421:280-288, 1976; McGuinness et al. Scand. J. Gastroneterol. 17:273-277, 1982; Ge et al., Br. J. Nutr. 70:333-345, (1993)]. A reduction or even exclusion of this feed-back regulation might be possible by the development of drug delivery systems which keep inhibitor(s) concentrated on a restricted area of the intestine, where drug liberation and subsequent absorption takes place. For a general review of more recent enzyme inhibitor strategies in the context of oral peptide drug delivery, see, e.g., Marschütz et al., Biomaterials 21:1499-1507, (2000).

The present invention provides coordinate administration methods and/or combinatorial formulations directed toward coordinate administration of a biologically active agent, including one or more JAM, occludin and claudin peptides,

proteins, analogs and mimetics, with an enzyme inhibitor. Since a variety of degradative enzymes are present in the mucosal environment, the prophylactic and therapeutic compositions and methods of the invention are readily modified to incorporate the addition or coadministration of an enzyme inhibitor, such as a protease inhibitor, with the biologically active agent (e.g., a physiologically active peptide or protein), to thereby improve bioavailability of the active agent. For example, in the case of therapeutically active peptides and proteins, one or more protease inhibiting agent(s) is/are optionally combined or coordinately administered in a formulation or method of the invention with one or more inhibitors of a proteolytic enzyme. In certain embodiments, the enzyme inhibitor is admixed with or bound to a common carrier with the biologically active agent. For example, an inhibitor of proteolytic enzymes may be incorporated in a therapeutic or prophylactic formulation of the invention to protect a biologically active protein or peptide from proteolysis, and thereby enhance bioavailability of the active protein or peptide.

Any inhibitor that inhibits the activity of an enzyme to protect the biologically active agent(s) may be usefully employed in the compositions and methods of the invention. Useful enzyme inhibitors for the protection of biologically active proteins and peptides include, for example, soybean trypsin inhibitor, pancreatic trypsin inhibitor, chymotrypsin inhibitor and trypsin and chrymotrypsin inhibitor isolated from potato (solanum tuberosum L.) tubers. A combination or mixtures of inhibitors may be employed. Additional inhibitors of proteolytic enzymes for use within the invention include ovomucoid-enzyme, gabaxate mesylate, alpha1-antitrypsin, aprotinin, amastatin, bestatin, puromycin, bacitracin, leupepsin, alpha2-macroglobulin, pepstatin and egg white or soybean trypsin inhibitor. These and other inhibitors can be used alone or in combination. The inhibitor(s) may be incorporated in or bound to a carrier, e.g., a hydrophilic polymer, coated on the surface of the dosage form which is to contact the nasal mucosa, or incorporated in the superficial phase of said surface, in combination with the biologically active agent or in a separately administered (e.g., pre-administered) formulation.

The amount of the inhibitor, e.g., of a proteolytic enzyme inhibitor that is optionally incorporated in the compositions of the invention will vary depending on (a) the properties of the specific inhibitor, (b) the number of functional groups present in the molecule (which may be reacted to introduce ethylenic unsaturation necessary for copolymerization with hydrogel forming monomers), and (c) the number of lectin

groups, such as glycosides, which are present in the inhibitor molecule. It may also depend on the specific therapeutic agent that is intended to be administered. Generally speaking, a useful amount of an enzyme inhibitor is from about 0.1 mg/ml to about 50 mg/ml, often from about 0.2 mg/ml to about 25 mg/ml, and more commonly from about 0.5 mg/ml to 5 mg/ml of the of the formulation (i.e., a separate protease inhibitor formulation or combined formulation with the inhibitor and biologically active agent).

With the necessary caveat of determining and considering possible toxic and other deleterious side effects, various inhibitors of proteases may be evaluated for use within the mucosal delivery methods and compositions of the invention. In the case of trypsin inhibition, suitable inhibitors may be selected from, e.g., aprotinin, BBI, soybean trypsin inhibitor, chicken ovomucoid, chicken ovoinhibitor, human pancreatic trypsin inhibitor, camostat mesilate, flavonoid inhibitors, antipain, leupeptin , p-aminobenzamidine, AEBSF, TLCK (tosyllysine chloromethylketone), APMSF, DFP, PMSF, and poly(acrylate) derivatives. In the case of chymotrypsin inhibition, suitable inhibitors may be selected from, e.g., aprotinin, BBI, soybean trypsin inhibitor, chymostatin, benzyloxycarbonyl-Pro-Phe-CHO, FK-448, chicken ovoinhibitor, sugar biphenylboronic acids complexes, DFP, PMSF, β-phenylpropionate, and poly(acrylate) derivatives. In the case of elastase inhibition, suitable inhibitors may be selected from, e.g., elastatinal, methoxysuccinyl-Ala-Ala-Pro-Val-chloromethylketone (MPCMK), BBI, soybean trypsin inhibitor, chicken ovoinhibitor, DFP, and PMSF. Other naturally occurring, endogenous enzyme inhibitors for additional known degradative enzymes present in the intranasal environment, or alternatively present in preparative materials for production of intranasal formulations, will be readily ascertained by those skilled in the art for incorporation within the methods and compositions of the invention.

Additional enzyme inhibitors for use within the invention are selected from a wide range of non-protein inhibitors that vary in their degree of potency and toxicity (see, e.g., L. Stryer, Biochemistry, WH Freeman and Company, NY, NY, 1988). As described in further detail below, immobilization of these adjunct agents to matrices or other delivery vehicles, or development of chemically modified analogues, may be readily implemented to reduce or even eliminate toxic effects, when they are encountered. Among this broad group of candidate enzyme inhibitors for use within

the invention are organophosphorous inhibitors, such as diisopropylfluorophosphate (DFP) and phenylmethylsulfonyl fluoride (PMSF), which are potent, irreversible inhibitors of serine proteases (e.g., trypsin and chymotrypsin). The additional inhibition of acetylcholinesterase by these compounds makes them highly toxic in uncontrolled delivery settings (L. Stryer, Biochemistry, WH Freeman and Company, NY, NY, 1988). Another candidate inhibitor, 4-(2-Aminoethyl)-benzenesulfonyl fluoride (AEBSF), has an inhibitory activity comparable to DFP and PMSF, but it is markedly less toxic. (4-Aminophenyl)-methanesulfonyl fluoride hydrochloride (APMSF) is another potent inhibitor of trypsin, but is toxic in uncontrolled settings. In contrast to these inhibitors, 4-(4-isopropylpiperadinocarbonyl)phenyl 1, 2,3,4,-tetrahydro-1-naphthoate methanesulphonate (FK-448) is a low toxic substance, representing a potent and specific inhibitor of chymotrypsin. The co-administration of this compound led to an enhanced intestinal absorption of insulin in rats and dogs, resulting in a decrease in blood glucose level. This increased bioavailability of insulin was found to be related to the inhibition of digestive enzymes, especially chymotrypsin (Fujii et al., J. Pharm. Pharmacol. 37:545-549, 1985). Further representatives of this non-protein group of inhibitor candidates, and also exhibiting low toxic risk, are camostat mesilate (N,N'-dimethyl carbamoylmethyl-p-(p '-guanidino-benzoyloxy)phenylacetate methane-sulphonate) (Yamamoto et al., Pharm. Res. 11:1496-1500, 1994) and Na-glycocholate (Yamamoto et al., Pharm. Res. 11:1496-1500, 1994; Okagava et al., Life Sci. 55:677-683, 1994).

Yet another type of enzyme inhibitory agent for use within the methods and compositions of the invention are amino acids and modified amino acids that interfere with enzymatic degradation of specific therapeutic compounds. For use in this context, amino acids and modified amino acids are substantially non-toxic and can be produced at a low cost. However, due to their low molecular size and good solubility, they are readily diluted and absorbed in mucosal environments. Nevertheless, under proper conditions, amino acids can act as reversible, competitive inhibitors of protease enzymes (see, e.g., McClellan et al., Biochim. Biophys Acta 613:160-167, 1980). Certain modified amino acids can display a much stronger inhibitory activity. A desired modified amino acid in this context is known as a 'transition-state' inhibitor. The strong inhibitory activity of these compounds is based on their structural similarity to a substrate in its transition-state geometry, while they are generally selected to have a much higher affinity for the active site of an enzyme than

the substrate itself. Transition-state inhibitors are reversible, competitive inhibitors. Examples of this type of inhibitor are α-aminoboronic acid derivatives, such as boro-leucine, boro-valine and boro-alanine. The boron atom in these derivatives can form a tetrahedral boronate ion that is believed to resemble the transition state of peptides during their hydrolysis by aminopeptidases. These amino acid derivatives are potent and reversible inhibitors of aminopeptidases and it is reported that boro-leucine is more than 100-times more effective in enzyme inhibition than bestatin and more than 1000-times more effective than puromycin (Hussain et al., Pharm. Res. 6:186-189, 1989). Another modified amino acid for which a strong protease inhibitory activity has been reported is N-acetylcysteine, which inhibits enzymatic activity of aminopeptidase N (Bernkop-Schnurch et al., Pharm. Res. 14:181-185, 1997). This adjunct agent also displays mucolytic properties that can be employed within the methods and compositions of the invention to reduce the effects of the mucus diffusion barrier (Bernkop-Schnurch et al., Pharm. Sci 2:361-363, 1996).

Still other useful enzyme inhibitors for use within the coordinate administration methods and combinatorial formulations of the invention may be selected from peptides and modified peptide enzyme inhibitors. An important representative of this class of inhibitors is the cyclic dodecapeptide, bacitracin, obtained from Bacillus licheniformis. Bacitracin A has a molecular mass of 1423 Da and shows remarkable resistance against the action of proteolytic enzymes like trypsin and pepsin (Hickey, R.J., Prog. Ind. Microbiol. 5:93-150, 1964). It has several biological properties inhibiting bacterial peptidoglycan synthesis, mammalian transglutaminase activity, and proteolytic enzymes such as aminopeptidase N. Because of its protease inhibitory activity, it has been used to inhibit the degradation of various therapeutic (poly)peptides, such as insulin, metkephamid, LH-RH, and buserelin (Yamamoto et al., Pharm. Res. 11:1496-1500, 1994; Langguth et al., J. Pharm. Pharmacol. 46:34-40, 1994; Raehs, et al., Pharm. Res. 5:689-693, 1988,). Besides its inhibitory activity, bacitracin also displays absorption-enhancing effects without leading to a serious intestinal mucosal damage (Gotoh et al., Biol. Pharm. Bull. 18:794-796, 1995).

Nevertheless, bacitracin may not be useful in certain uncontrolled delivery settings due to its established nephrotoxicity. To date, it has almost exclusively been used in veterinary medicine and as a topical antibiotic in the treatment of infections in

man. Covalent linkage of bacitracin to a mucoadhesive polymer (carbomer) has been shown to conserve the inhibitory activity of the compound within the carrier matrix (Bernkop-Schnurch et al., Pharm. Res. 14:181-185, 1997).

In addition to these types of peptides, certain dipeptides and tripeptides display weak, non-specific inhibitory activity towards some proteases (Langguth et al., J. Pharm. Pharmacol. 46:34-40, 1994). By analogy with amino acids, their inhibitory activity can be improved by chemical modifications. For example, phosphinic acid dipeptide analogues are also 'transition-state' inhibitors with a strong inhibitory activity towards aminopeptidases. They have reportedly been used to stabilize nasally administered leucine enkephalin (Hussain et al., Pharm. Res. 9:626-628, 1992). Another example of a transition-state analogue is the modified pentapeptide pepstatin (McConnell et al., J. Med. Chem. 34:2298-2300, 1991), which is a very potent inhibitor of pepsin. Structural analysis of pepstatin, by testing the inhibitory activity of several synthetic analogues, demonstrated the major structure-function characteristics of the molecule responsible for the inhibitory activity (McConnell et al., J. Med. Chem. 34:2298-2300, 1991). Similar analytic methods can be readily applied to prepare modified amino acid and peptide analogs for blockade of selected, intranasal degradative enzymes.

Another special type of modified peptide includes inhibitors with a terminally located aldehyde function in their structure. For example, the sequence benzyloxycarbonyl-Pro-Phe-CHO, which fulfill the known primary and secondary specificity requirements of chymotrypsin, has been found to be a potent reversible inhibitor of this target proteinase (Walker et al., Biochem. J. 321-323, 1993). The chemical structures of further inhibitors with a terminally located aldehyde function, e.g. antipain, leupeptin, chymostatin and elastatinal, are also known in the art, as are the structures of other known, reversible, modified peptide inhibitors, such as phosphoramidon, bestatin, puromycin and amastatin

Due to their comparably high molecular mass, polypeptide protease inhibitors are more amenable than smaller compounds to concentrated delivery in a drug-carrier matrix. The advantages of a slow release carrier system for delivery of enzyme inhibitors have been discussed by Kimura et al. (Biol. Pharm. Bull. 19:897-900, 1996). In this study a mucoadhesive delivery system exhibited a desired release rate of the protease inhibitor aprotinin of approximately 10% per hour, which was almost synchronous with the release rate of a polypeptide drug. *In vivo* studies with this

delivery system showed an improved bioavailability of the drug (id.) For this reason, and due to their low toxicity and strong inhibitory activity, polypeptide protease inhibitors will often be selected for use within the methods and compositions of the invention.

Additional agents for protease inhibition within the formulations and methods of the invention involve the use of complexing agents. These agents mediate enzyme inhibition by depriving the intranasal environment (or preparative or therapeutic composition) of divalent cations which are co-factors for many proteases. For instance, the complexing agents EDTA and DTPA as coordinately administered or combinatorially formulated adjunct agents, in suitable concentration, will be sufficient to inhibit selected proteases to thereby enhance intranasal delivery of biologically active agents according to the invention. Further representatives of this class of inhibitory agents are EGTA, 1,10-phenanthroline and hydroxychinoline (Ikesue et al., Int. J. Pharm. 95:171-9, 1993; Garner et al., Biochemistry 13:3227-3233, 1974; Sangadala et al., J. Biol. Chem. 269:10088-10092, 1994; Mizuma et al., Biochim. Biophys. Acta. 1335:111-119, 1997). In addition, due to their propensity to chelate divalent cations, these and other complexing agents are useful within the invention as direct, absorption-promoting agents (see, e.g., Lee, V.H.L., J. Control Release 13:213-334, 1990,).

As noted in more detail elsewhere herein, it is also contemplated to use various polymers, particularly mucoadhesive polymers, as enzyme inhibiting agents within the coordinate administration, multi-processing and/or combinatorial formulation methods and compositions of the invention. For example, poly(acrylate) derivatives, such as poly(acrylic acid) and polycarbophil, can affect the activity of various proteases, including trypsin, chymotrypsin. The inhibitory effect of these polymers may also be based on the complexation of divalent cations such as Ca^{2+} and Zn^{2+} (Lueβen et al., Pharm. Res. 12:1293-1298, 1995). It is further contemplated that these polymers may serve as conjugate partners or carriers for additional enzyme inhibitory agents, as described above. For example, a chitosan-EDTA conjugate has been developed and is useful within the invention that exhibits a strong inhibitory effect towards the enzymatic activity of zinc-dependent proteases. The mucoadhesive properties of polymers following covalent attachment of other enzyme inhibitors in this context are not expected to be substantially compromised, nor is the general

utility of such polymers as a delivery vehicle for biologically active agents within the invention expected to be diminished. On the contrary, the reduced distance between the delivery vehicle and mucosal surface afforded by the mucoadhesive mechanism will minimize presystemic metabolism of the active agent, while the covalently bound enzyme inhibitors remain concentrated at the site of drug delivery, minimizing undesired dilution effects of inhibitors as well as toxic and other side effects caused thereby. In this manner, the effective amount of a coordinately administered enzyme inhibitor can be reduced due to the exclusion of dilution effects.

More recent research efforts in the area of protease inhibition for enhanced delivery of peptide and protein therapeutics has focused on covalent immobilization of protease inhibitors on mucoadhesive polymers used as drug carrier matrices (see, e.g., Bernkop-Schnurch et al., Drug Dev. Ind. Pharm. 23:733-40, 1997; Bernkop-Schnurch et al., J. Control. Rel. 47:113-21, 1997; Bernkop-Schnurch et al., J. Drug Targ. 7:55-63, 1999). In conjunction with these teachings, the invention provides in more detailed aspects an enzyme inhibitor formulated with a common carrier or vehicle for intranasal delivery of a biologically active agent. Optionally, the enzyme inhibitor is covalently linked to the carrier or vehicle. In certain embodiments, the carrier or vehicle is a biodegradable polymer, for example, a bioadhesive polymer. Thus, for example, a protease inhibitor, such as Bowman-Birk inhibitor (BBI), displaying an inhibitory effect towards trypsin and α-chymotrypsin (Birk Y. Int. J. Pept. Protein Res. 25:113-31, 1985), or elastatinal, an elastase-specific inhibitor of low molecular size, may be covalently linked to a mucoadhesive polymer as described herein. The resulting polymer-inhibitor conjugate exhibits substantial utility as an intranasal delivery vehicle for biologically active agents according to the methods and compositions of the invention.

Exemplary mucoadhesive polymer-enzyme inhibitor complexes that are useful within the mucosal formulations and methods of the invention include, but are not limited to: Carboxymethylcellulose-pepstatin (with anti-pepsin activity); Poly(acrylic acid)-Bowman-Birk inhibitor (anti-chymotrypsin); Poly(acrylic acid)-chymostatin (anti-chymotrypsin); Poly(acrylic acid)-elastatinal (anti-elastase); Carboxymethylcellulose-elastatinal (anti-elastase); Polycarbophil—elastatinal (anti-elastase); Chitosan—antipain (anti-trypsin); Poly(acrylic acid)—bacitracin (anti-aminopeptidase N); Chitosan—EDTA (anti-aminopeptidase N, anti-carboxypeptidase

A); Chitosan—EDTA—antipain (anti-trypsin, anti-chymotrypsin, anti-elastase) (see, e.g., Bernkop-Schnürch, J. Control. Rel. 52:1-16, 1998).

MUCOLYTIC AND MUCUS-CLEARING AGENTS AND METHODS

Effective delivery of biotherapeutic agents via intranasal administration must take into account the decreased drug transport rate across the protective mucus lining of the nasal mucosa, in addition to drug loss due to binding to glycoproteins of the mucus layer. Normal mucus is a viscoelastic, gel-like substance consisting of water, electrolytes, mucins, macromolecules, and sloughed epithelial cells. It serves primarily as a cytoprotective and lubricative covering for the underlying mucosal tissues. Mucus is secreted by randomly distributed secretory cells located in the nasal epithelium and in other mucosal epithelia. The structural unit of mucus is mucin. This glycoprotein is mainly responsible for the viscoelastic nature of mucus, although other macromolecules may also contribute to this property. In airway mucus, such macromolecules include locally produced secretory IgA, 1gM, IgE, lysozyme, and bronchotransferrin, which also play an important role in host defense mechanisms.

The thickness of mucus varies from organ to organ and between species. However, mucin glycoproteins obtained from different sources have similar overall amino acid and protein/carbohydrate compositions, although the molecular weight may vary over a wide. Mucin consists of a large protein core with oligosaccharide side-chains attached through the O-glycosidic linkage of galactose or N-acetyl glucosamine to hydroxyl groups of serine and threonine residues. Either sialic acid or L-fucose forms the terminal group of the side chain oligosaccharides with sialic acid (negatively charged at pH greater than 2.8) forming 50 to 60% of the terminal groups. The presence of cysteine in the end regions of the mucin core facilitates cross-linking of mucin molecules via disulfide bridge formation.

The presence of a mucus layer that coats all epithelial surfaces has been largely overlooked in the elucidation of epithelial penetration enhancement mechanisms to date. This is partly because the role of mucus in the absorption of peptide and protein drugs has not yet been well established. However, for these and other drugs exhibiting a comparatively high molecular mass, the mucus layer covering the nasal mucosal surfaces may represent an almost insurmountable barrier.

According to the conventional formula for calculation of the diffusion coefficient, in which the radius of the molecule indirectly correlates with the diffusion coefficient, the mucus barrier increases tremendously for polypeptide drugs. Studies focusing on this so called 'diffusion barrier' have demonstrated that proteins of a molecular mass greater than approximately 5 kDa exhibit minimal or no permeation into mucus layers (Allen, et al., 'Mucus Medicine and Biology', E. N. Elder, J. B. Elstein (eds.) p. 115, Vol. 144, Plenum Press, New York, 1982; Bernkop-Schnurch., Pharm. Sci. 2:361, 1996).

The coordinate administration methods of the instant invention optionally incorporate effective mucolytic or mucus-clearing agents, which serve to degrade, thin or clear mucus from intranasal mucosal surfaces to facilitate absorption of intranasally administered biotherapeutic agents. Within these methods, a mucolytic or mucus-clearing agent is coordinately administered as an adjunct compound to enhance intranasal delivery of the biologically active agent. Alternatively, an effective amount of a mucolytic or mucus-clearing agent is incorporated as a processing agent within a multi-processing method of the invention, or as an additive within a combinatorial formulation of the invention, to provide an improved formulation that enhances intranasal delivery of biotherapeutic compounds by reducing the barrier effects of intranasal mucus.

A variety of mucolytic or mucus-clearing agents are available for incorporation within the methods and compositions of the invention (see, e.g., Lee, et al., Crit. Rev. Ther. Drug Carrier Syst. 8:91-192, 1991; Bernkop-Schnurch et al., Arzneimittelforschung, 49:799-803, 1999). Based on their mechanisms of action, mucolytic and mucus clearing agents can often be classified into the following groups: proteases (e.g., pronase, papain) that cleave the protein core of mucin glycoproteins; sulfhydryl compounds that split mucoprotein disulfide linkages; and detergents (e.g., Triton X-100, Tween 20) that break non-covalent bonds within the mucus (see, e.g., Allen, A. in 'Physiology of the Gastrointestinal Tract. L.R. Johnson (ed.), p. 617, Raven Press, New York, 1981). Additional compounds in this context include, but are not limited to, bile salts and surfactants, for example, sodium deoxycholate, sodium taurodeoxycholate, sodium glycocholate, and lysophosphatidylcholine.

The effectiveness of bile salts in causing structural breakdown of mucus is in the order deoxycholate > taurocholate > glycocholate. Other effective agents that

reduce mucus viscosity or adhesion to enhance intranasal delivery according to the methods of the invention include, e.g., short-chain fatty acids, and mucolytic agents that work by chelation, such as N-acylcollagen peptides, bile acids, and saponins (the latter function in part by chelating Ca^{2+} and/or Mg^{2+} which play an important role in maintaining mucus layer structure).

Additional mucolytic agents for use within the methods and compositions of the invention include N-acetyl-L-cysteine (ACS), a potent mucolytic agent that reduces both the viscosity and adherence of bronchopulmonary mucus and is reported to modestly increase nasal bioavailability of human growth hormone in anesthetized rats (from 7.5 to 12.2%) (O'Hagen et al., Pharm. Res., 7:772, 1990). These and other mucolytic or mucus-clearing agents are contacted with the nasal mucosa, typically in a concentration range of about 0.2 to 20 mM, coordinately with administration of the biologically active agent, to reduce the polar viscosity and/or elasticity of intranasal mucus.

Still other mucolytic or mucus-clearing agents may be selected from a range of glycosidase enzymes, which are able to cleave glycosidic bonds within the mucus glycoprotein. α-amylase and ß-amylase are representative of this class of enzymes, although their mucolytic effect may be limited (Leiberman, J., Am. Rev. Respir. Dis. 97:662, 1967,). In contrast, bacterial glycosidases which allow these microorganisms to permeate mucus layers of their hosts (Corfield et al, Glycoconjugate J. 10:72, 1993,) are highly mucolytic active.

For selecting mucolytic agents for use within the methods and compositions of the invention, it is important to consider the chemical nature of both the mucolytic (or mucus-clearing) and biologically active agents. For example, the proteolytic enzyme pronase exhibits a very strong mucolytic activity at pH 5.0, as well as at pH 7.2. In contrast, the protease papain exhibited substantial mucolytic activity at pH 5.0, but no detectable mucolytic activity at pH 7.2. The reason for these differences in activity are explained in part by the distinct pH-optimum for papain, reported to be pH 5 (Karlson, P., Biochemie, Thieme, Verlag, Stuttgart, New York, 1984,). Thus, mucolytic and other enzymes for use within the invention are typically delivered in formulations having a pH at or near the pH optimum of the subject enzyme.

With respect to chemical characterization of the biologically active agent, one notable concern is the vulnerability of peptide and protein molecules to the degradative activities of proteases and sulfhydryl. In particular, peptide and protein

drugs can be attacked by different types of mucolytic agents. In one study, the mucolytic proteases pronase and papain (which each are endopeptidases that cleave at a high number of bonds) were shown to completely degrade insulin within 2-3h at pH 7.2 (Bernkop-Schnurch et al., Arzneimittelforschung, 49:799-803, 1999,). In contrast, at pH 2.5 insulin was not at all, or only slightly, degraded by pronase and papain, which can be explained by the pH optimum of both enzymes being far away from pH 2.5. Whereas pronase represents an unusually non-specific protease, papain cleaves after Arg, Lys, Leu, and Gly (Karlson, P., Biochemie, Thieme, Verlag, Stuttgart, New York, 1984), which are all included in the primary structure of insulin and serve as an additional guide to selection of mucolytic and mucus-clearing agents within the invention.

The presence and number of cysteine residues and disulfide bonds in peptide and protein therapeutics are also important factors to consider in selecting mucolytic or mucus-clearing agents within the invention. When insulin, which displays three disulfide bonds within its molecular structure, is incubated with di-thiothreitol or N-acetylcysteine, there is a rapid degradation of the insulin polypeptide at pH 7.2. A substantially lower degree of degradation at pH 2.5 is attributed to the relatively low amount of reactive thiolate anions (responsible for nucleophilic attack on disulfide bonds) at this pH value (Bernkop-Schnurch et al., Arzneimittelforschung, 49:799-803, 1999).

Whereas it is generally contraindicated to use general proteases such as pronase or papain in combination with peptide or protein drugs, the practical use of more specific proteases can be undertaken according to the above principals, as can the use of sulfhydryl compounds. For therapeutic polypeptides that exhibit no cysteine moieties within their primary structure (e.g. cyclosporin), the use of sulfhydryl compounds is not problematic. Moreover, even for protein drugs bearing disulfide bonds the use of sulfhydryl compounds can be achieved, particularly where the disulfide bonds are not accessible for thiol attack due to the conformation of the protein, they should remain stable in the presence of this type of mucolytic agents.

For combinatorial use with most biologically active agents within the invention, including peptide and protein therapeutics, non-ionogenic detergents are generally also useful as mucolytic or mucus-clearing agents. These agents typically will not modify or substantially impair the activity of therapeutic polypeptides.

CILIOSTATIC AGENTS AND METHODS

Because the self-cleaning capacity of certain mucosal tissues (e.g., nasal mucosal tissues) by mucociliary clearance is necessary as a protective function (e.g., to remove dust, allergens, and bacteria), it has been generally considered that this function should not be substantially impaired by mucosal medications. Mucociliary transport in the respiratory tract is a particularly important defense mechanism against infections (Wasserman., J. Allergy Clin. Immunol. 73:17-19, 1984). To achieve this function, ciliary beating in the nasal and airway passages moves a layer of mucus along the mucosa to removing inhaled particles and microorganisms. During chronic bronchitis and chronic sinusitis, tracheal and nasal mucociliary clearance are often impaired (Wanner., Am. Rev. Respir. Dis. 116:73-125, 1977). This is presumably due to either excess secretion (Dulfano, et al., Am. Rev. Respir. Dis. 104:88-98, 1971), increased viscosity of mucus (Chen, et al., J. Lab. Clin. Med. 91:423-431, 1978), alterations in ciliary activity caused by decreased beat frequency (Puchelle et al., Biorheology 21:265-272, 1984,), loss of portions of the ciliated epithelium (Chodosh et al., Am. Rev. Respir. Dis. 104:888-898, 1971), or to a combination of these factors. Decreased clearance presumably favors bacterial colonization of respiratory mucosal surfaces, predisposing the subject to infection. The ability to interfere with this host defense system may contribute significantly to a pathological organism's virulence.

Various reports show that mucociliary clearance can be impaired by mucosally administered drugs, as well as by a wide range of formulation additives including penetration enhancers and preservatives. For example, ethanol at concentrations greater than 2% has been shown to reduce the *in vitro* ciliary beating frequency.. This may be mediated in part by an increase in membrane permeability that indirectly enhances flux of calcium ion which, at high concentration, is ciliostatic, or by a direct effect on the ciliary axoneme or actuation of regulatory proteins involved in a ciliary arrest response. Exemplary preservatives (methyl-p-hydroxybenzoate (0.02% and 0.15%), propyl-p-hydroxybenzoate (0.02%), and chlorobutanol (0.5%)) reversibly inhibit ciliary activity in a frog palate model. Other common additives (EDTA (0.1%), benzalkoniuin chloride (0.01%), chlorhexidine (0.01%), phenylinercuric nitrate (0.002%), and phenylmercuric borate (0.002%), have been reported to inhibit

mucociliary transport irreversibly. In addition, several penetration enhancers including STDHF, laureth-9, deoxycholate, deoxycholic acid, taurocholic acid, and glycocholic acid have been reported to inhibit ciliary activity in model systems.

Despite the potential for adverse effects on mucociliary clearance attributed to ciliostatic factors, ciliostatic agents nonetheless find use within the methods and compositions of the invention to increase the residence time of mucosally (e.g., intranasally) administered JAM, occludin and claudin peptides, proteins, analogs and mimetics, and other biologically active agents disclosed herein. In particular, the delivery these agents within the methods and compositions of the invention is significantly enhanced in certain aspects by the coordinate administration or combinatorial formulation of one or more ciliostatic agents that function to reversibly inhibit ciliary activity of mucosal cells, to provide for a temporary, reversible increase in the residence time of the mucosally administered active agent(s). For use within these aspects of the invention, the foregoing ciliostatic factors, either specific or indirect in their activity, are all candidates for successful employment as ciliostatic agents in appropriate amounts (depending on concentration, duration and mode of delivery) such that they yield a transient (i.e., reversible) reduction or cessation of mucociliary clearance at a mucosal site of administration to enhance delivery of JAM, occludin and claudin peptides, proteins, analogs and mimetics, and other biologically active agents disclosed herein, without unacceptable adverse side effects.

Within more detailed aspects, a specific ciliostatic factor is employed in a combined formulation or coordinate administration protocol with one or more JAM, occludin and/or claudin peptides, proteins, analogs and mimetics, and/or other biologically active agents disclosed herein. Various bacterial ciliostatic factors isolated and characterized in the literature may be employed within these embodiments of the invention. For example, Hingley, et al. (Infection and Immunity. 51:254-262, 1986) have recently identified ciliostatic factors from the bacterium *Pseudomonas aeruginosa*. These are heat-stable factors released by Pseudomonas aeruginosa in culture supernatants that have been shown to inhibit ciliary function in epithelial cell cultures. Exemplary among these cilioinhibitory components are a phenazine derivative, a pyo compound (2-alkyl-4-hydroxyquinolines), and a rhamnolipid (also known as a hemolysin). Inhibitory concentrations of these and other active components were established by quantitative measures of ciliary motility and beat frequency. The pyo compound produced ciliostasis at concentrations of 50

μg/ml and without obvious ultrastructural lesions. The phenazine derivative also inhibited ciliary motility but caused some membrane disruption, although at substantially greater concentrations of 400 μg/ml. Limited exposure of tracheal explants to the rhamnolipid resulted in ciliostasis which was associated with altered ciliary membranes. More extensive exposure to rhamnolipid was associated with removal of dynein arms from axonemes. It is proposed that these and other bacterial ciliostatic factors have evolved to enable *P. aeruginosa* to more easily and successfully colonize the respiratory tract of mammalian hosts. On this basis, respiratory bacteria are useful pathogens for identification of suitable, specific ciliostatic factors for use within the methods and compositions of the invention.

Several methods are available to measure mucociliary clearance for evaluating the effects and uses of ciliostatic agents within the methods and compositions of the invention. Nasal mucociliary clearance can be measured by monitoring the disappearance of visible tracers such as India ink, edicol orange powder, and edicol supra orange. These tracers are followed either by direct observation or with the aid of posterior rhinoscopy or a binocular operating microscope. This method simply measures the time taken by a tracer to travel a definite distance. In more modern techniques, radiolabeled tracers are administered as an aerosol and traced by suitably collimated detectors. Alternatively, particles with a strong taste like saccharin can be placed in the nasal passage and assayed to determine the time before the subject first perceives the taste is used as an indicator of mucociliary clearance.

Additional assays are known in the art for measuring ciliary beat activity. For example, a laser light scattering technique to measure tracheobronchial mucociliary activity is based on mono-chromaticity, coherence, and directionality of laser light. Ciliary motion is measured as intensity fluctuations due to the interference of Doppler-shifted scattered light. The scattered light from moving cilia is detected by a photomultiplier tube and its frequency content analyzed by a signal correlator yielding an autocorrelation function of the detected photocurrents. In this way, both the frequency and synchrony of beating cilia can be measured continuously. Through fiberoptic rhinoscopy, this method also allows the measurement of ciliary activity in the peripheral parts of the nasal passages.

In vitro assays for evaluating ciliostatic activity of formulations within the invention are also available. For example, a commonly used and accepted assay in this context is a rabbit tracheal explant system (Gabridge et al., Pediatr. Res. 1:31-35,

1979; Chandler et al., Infect. Immun. 29:1111-1116, 1980). Other assay systems measure the ciliary beat frequency of a single cell or a small number of cells (Kennedy et al., Exp. Cell Res. 135:147-156, 1981; Rutland et al., Lancet ii 564-565, 1980; Verdugo, et al., Pediatr. Res. 13:131-135, 1979).

SURFACE ACTIVE AGENTS AND METHODS

Within more detailed aspects of the invention, one or more membrane penetration-enhancing agents may be employed within a mucosal delivery method or formulation of the invention to enhance mucosal delivery of JAM, occludin and claudin peptides, proteins, analogs and mimetics, and other biologically active agents disclosed herein. Membrane penetration enhancing agents in this context can be selected from: (i) a surfactant, (ii) a bile salt, (ii) a phospholipid additive, mixed micelle, liposome, or carrier, (iii) an alcohol, (iv) an enamine, (v) an NO donor compound, (vi) a long-chain amphipathic molecule (vii) a small hydrophobic penetration enhancer; (viii) sodium or a salicylic acid derivative; (ix) a glycerol ester of acetoacetic acid (x) a cyclodextrin or beta-cyclodextrin derivative, (xi) a medium-chain fatty acid, (xii) a chelating agent, (xiii) an amino acid or salt thereof, (xiv) an N-acetylamino acid or salt thereof, (xv) an enzyme degradative to a selected membrane component, (ix) an inhibitor of fatty acid synthesis, or (x) an inhibitor of cholesterol synthesis; or (xi) any combination of the membrane penetration enhancing agents recited in (i)-(x)

Certain surface-active agents are readily incorporated within the mucosal delivery formulations and methods of the invention as mucosal absorption enhancing agents. These agents, which may be coordinately administered or combinatorially formulated with JAM, occludin and claudin peptides, proteins, analogs and mimetics, and other biologically active agents disclosed herein, may be selected from a broad assemblage of known surfactants. Surfactants, which generally fall into three classes: (1) nonionic polyoxyethylene ethers; (2) bile salts such as sodium glycocholate (SGC) and deoxycholate (DOC); and (3) derivatives of fusidic acid such as sodium taurodihydrofusidate (STDHF). The mechanisms of action of these various classes of surface active agents typically include solubilization of the biologically active agent. For proteins and peptides which often form aggregates, the surface active properties

of these absorption promoters can allow interactions with proteins such that smaller units such as surfactant coated monomers may be more readily maintained in solution. These monomers are presumably more transportable units than aggregates. A second potential mechanism is the protection of the peptide or protein from proteolytic degradation by proteases in the mucosal environment. Both bile salts and some fusidic acid derivatives reportedly inhibit proteolytic degradation of proteins by nasal homogenates at concentrations less than or equivalent to those required to enhance protein absorption. This protease inhibition may be especially important for peptides with short biological half-lives.

DEGRADATION ENZYMES AND INHIBITORS OF FATTY ACID AND CHOLESTEROL SYNTHESIS

In related aspects of the invention, JAM, occludin and claudin peptides, proteins, analogs and mimetics, and other biologically active agents for mucosal administration are formulated or coordinately administered with a penetration enhancing agent selected from a degradation enzyme, or a metabolic stimulatory agent or inhibitor of synthesis of fatty acids, sterols or other selected epithelial barrier components (see, e.g., U.S. Patent No. 6,190,894). In one embodiment, known enzymes that act on mucosal tissue components to enhance permeability are incorporated in a combinatorial formulation or coordinate administration method of instant invention, as processing agents within the multi-processing methods of the invention. For example, degradative enzymes such as phospholipase, hyaluronidase, neuraminidase, and chondroitinase may be employed to enhance mucosal penetration of JAM, occludin and claudin peptides, proteins, analogs and mimetics, and other biologically active agents (see, e.g., Squier Brit. J. Dermatol. 111:253-264, 1984; Aungst and Rogers Int. J. Pharm. 53:227-235, 1989), without causing irreversible damage to the mucosal barrier. In one embodiment, chondroitinase is employed within a method or composition as provided herein to alter glycoprotein or glycolipid constituents of the permeability barrier of the mucosa, thereby enhancing mucosal absorption of JAM, occludin and/or claudin peptides, proteins, analogs and mimetics, and other biologically active agents disclosed herein.

With regard to inhibitors of synthesis of mucosal barrier constituents, it is noted that free fatty acids account for 20-25% of epithelial lipids by weight. Two rate limiting enzymes in the biosynthesis of free fatty acids are acetyl CoA carboxylase and fatty acid synthetase. Through a series of steps, free fatty acids are metabolized into phospholipids. Thus, inhibitors of free fatty acid synthesis and metabolism for use within the methods and compositions of the invention include, but are not limited to, inhibitors of acetyl CoA carboxylase such as 5-tetradecyloxy-2-furancarboxylic acid (TOFA); inhibitors of fatty acid synthetase; inhibitors of phospholipase A such as gomisin A, 2-(p-amylcinnamyl)amino-4-chlorobenzoic acid, bromophenacyl bromide, monoalide, 7,7-dimethyl-5,8-eicosadienoic acid, nicergoline, cepharanthine, nicardipine, quercetin, dibutyryl-cyclic AMP, R-24571, N-oleoylethanolamine, N-(7-nitro-2,1,3-benzoxadiazol-4-yl) phosphostidyl serine, cyclosporine A, topical anesthetics, including dibucaine, prenylamine, retinoids, such as all-trans and 13-cis-retinoic acid, W-7, trifluoperazine, R-24571 (calmidazolium), 1-hexadocyl-3-trifluoroethyl glycero-sn-2-phosphomenthol (MJ33); calcium channel blockers including nicardipine, verapamil, diltiazem, nifedipine, and nimodipine; antimalarials including quinacrine, mepacrine, chloroquine and hydroxychloroquine; beta blockers including propanalol and labetalol; calmodulin antagonists; EGTA; thimersol; glucocorticosteroids including dexamethasone and prednisolone; and nonsteroidal antiinflammatory agents including indomethacin and naproxen.

Free sterols, primarily cholesterol, account for 20-25% of the epithelial lipids by weight. The rate limiting enzyme in the biosynthesis of cholesterol is 3-hydroxy-3-methylglutaryl (HMG) CoA reductase. Inhibitors of cholesterol synthesis for use within the methods and compositions of the invention include, but are not limited to, competitive inhibitors of (HMG) CoA reductase, such as simvastatin, lovastatin, fluindostatin (fluvastatin), pravastatin, mevastatin, as well as other HMG CoA reductase inhibitors, such as cholesterol oleate, cholesterol sulfate and phosphate, and oxygenated sterols, such as 25-OH-- and 26-OH-- cholesterol; inhibitors of squalene synthetase; inhibitors of squalene epoxidase; inhibitors of DELTA7 or DELTA24 reductases such as 22,25-diazacholesterol, 20,25-diazacholestenol, AY9944, and triparanol.

Each of the inhibitors of fatty acid synthesis or the sterol synthesis inhibitors may be coordinately administered or combinatorially formulated with one or more JAM, occludin and claudin peptides, proteins, analogs and mimetics, and other

biologically active agents disclosed herein to achieve enhanced epithelial penetration of the active agent(s). An effective concentration range for the sterol inhibitor in a therapeutic or adjunct formulation for mucosal delivery is generally from about 0.0001% to about 20% by weight of the total, more typically from about 0.01% to about 5%.

NITRIC OXIDE DONOR AGENTS AND METHODS

Within other related aspects of the invention, a nitric oxide (NO) donor is selected as a membrane penetration-enhancing agent to enhance mucosal delivery of one or more JAM, occludin and claudin peptides, proteins, analogs and mimetics, and other biologically active agents disclosed herein. Recently, Salzman et al. (Am. J. Physiol. 268:G361-G373, 1995) reported that NO donors increase the permeability of water-soluble compounds across Caco-2 cell monolayers with neither loss of cell viability nor lactate dehydrogenase (LDH) release. In addition, Utoguchi et al. (Pharm. Res. 15:870-876, 1998) demonstrated that the rectal absorption of insulin was remarkably enhanced in the presence of NO donors, with attendant low cytotoxicity as evaluated by the cell detachment and LDH release studies in Caco-2 cells.

Various NO donors are known in the art and are useful in effective concentrations within the methods and formulations of the invention. Exemplary NO donors include, but are not limited to, nitroglycerine, nitropruside, NOC5 [3-(2-hydroxy-1-(methyl-ethyl)-2-nitrosohydrazino)-1-propanamine], NOC12 [*N*-ethyl-2-(1-ethyl-hydroxy-2-nitrosohydrazino)-ethanamine], SNAP [S-nitroso-N-acetyl-DL-penicillamine], NORI and NOR4. Efficacy of these and other NO donors, as well as other mucosal delivery-enhancing agents disclosed herein, for enhancing mucosal delivery of JAM, occludin and claudin peptides, proteins, analogs and mimetics, and other biologically active agents can be evaluated routinely according to known efficacy and cytotoxicity assay methods (e.g., involving control coadministration of an NO scavenger, such as carboxy-PIIO) as described by Utoguchi et al., Pharm. Res. 15:870-876, 1998.

Within the methods and compositions of the invention, an effective amount of a selected NO donor is coordinately administered or combinatorially formulated with one or more JAM, occludin and claudin peptides, proteins, analogs and mimetics,

and/or other biologically active agents disclosed herein, into or through the mucosal epithelium.

ADDITIONAL AGENTS FOR MODULATING EPITHELIAL JUNCTION STRUCTURE AND/OR PHYSIOLOGY

In addition to JAM, occludin and claudin peptides, proteins, analogs and mimetics, additional agents for modulating epithelial junctional physiology and/or structure are contemplated for use within the methods and formulations of the invention. Epithelial tight junctions are generally impermeable to molecules with radii of approximately 15 angstroms, unless treated with junctional physiological control agents that stimulate substantial junctional opening as provided within the instant invention. Among the "secondary" tight junctional regulatory components that will serve as useful targets for secondary physiological modulation within the methods and compositions of the invention, the ZO1-ZO2 heterodimeric complex has shown itself amenable to physiological regulation by exogenous agents that can readily and effectively alter paracellular permeability in mucosal epithelia. On such agent that has been extensively studied is the bacterial toxin from *Vibrio cholerae* known as the "zonula occludens toxin" (ZOT). This toxin mediates increased intestinal mucosal permeability and causes disease symptoms including diarrhea in infected subjects (Fasano et al, Proc. Nat. Acad. Sci., USA 8:5242-5246, 1991; Johnson et al, J. Clin. Microb. 31/3:732-733, 1993; and Karasawa et al, FEBS Let. 106:143-146, 1993). When tested on rabbit ileal mucosa, ZOT increased the intestinal permeability by modulating the structure of intercellular tight junctions. More recently, it has been found that ZOT is capable of reversibly opening tight junctions in the intestinal mucosa (see, e.g., WO 96/37196; U.S. Pat. No.s 5,945,510; 5,948,629; 5,912,323; 5,864,014; 5,827,534; 5,665,389). It has also been reported that ZOT is capable of reversibly opening tight junctions in the nasal mucosa (U.S. Pat No. 5,908,825). Thus, ZOT and other agents that modulate the ZO1-ZO2 complex will be combinatorially formulated or coordinately administered with one or more JAM, occludin and claudin peptides, proteins, analogs and mimetics, and/or other biologically active agents disclosed herein.

Within the methods and compositions of the invention, ZOT, as well as various analogs and mimetics of ZOT that function as agonists or antagonists of ZOT activity, are useful for enhancing intranasal delivery of biologically active agents—by increasing paracellular absorption into and across the nasal mucosa. In this context, ZOT typically acts by causing a structural reorganization of tight junctions marked by altered localization of the junctional protein ZO1. Within these aspects of the invention, ZOT is coordinately administered or combinatorially formulated with the biologically active agent in an effective amount to yield significantly enhanced absorption of the active agent, by reversibly increasing nasal mucosal permeability without substantial adverse side effects

Suitable methods for determining ZOT biological activity may be selected from a variety of known assays, e.g., involving assaying for a decrease of tissue or cell culture resistance (Rt) using Ussing chambers (e.g., as described by Fasano et al, Proc. Natl. Acad. Sci., USA, 8:5242-5246, 1991,), assaying for a decrease of tissue resistance (Rt) of intestinal epithelial cell monolayers in Ussing chambers; or directly assaying enhancement of absorption of a therapeutic agent across a mucosal surface *in vivo*.

In addition to ZOT, various other tight junction modulatory agents can be employed within the methods and compositions of the invention that mimic the activity of ZOT by reversibly increasing mucosal epithelial paracellular permeability. These include specific binding or blocking agents, such as antibodies, antibody fragments, peptides, peptide mimetics, bacterial toxins and other agents that serve as agonists or antagonists of ZOT activity, or which otherwise alter physiology of the ZO1-ZO2 complex (e.g., by blocking dimerization). Naturally, these additional regulatory agents include peptide analogs, including site-directed mutant variants, of the native ZOT protein, as well as truncated active forms of the protein and peptide mimetics that model functional domains or active sites of the native protein. In addition, these agents include a native mammalian protein "zonulin", which has been proposed to be an endogenous regulator of tight junctional physiology similar in both structural and functional aspects to ZOT (see, e.g., WO 96/37196; WO 00/07609; U.S. Pat. No.s 5,945,510; 5,948,629; 5,912,323; 5,864,014; 5,827,534; 5,665,389,), which therefore suggests that ZOT is a convergent evolutionary development of *Vibrio cholerae* patterned after the endogenous mammalian zonulin regulatory

mechanism to facilitate host entry. Both zonulin and ZOT are proposed to bind a specific membrane receptor, designated "ZOT receptor" (see, e.g., U.S. Pat. No. 5,864,014; 5,912,323; and 5,948,629), which can be used within the invention to screen for additional agonists and antagonists to ZOT and zonulin activity for regulation of tight junctional physiology. In this context, structure-function analysis of the ZOT receptor, and comparisons between ZOT and zonulin, will guide production and selection of specific binding or blocking agents, (e.g., antibodies, antibody fragments, peptides, peptide mimetics, additional bacterial toxins and other agents) to serve as ZOT or zonulin agonists or antagonists, for example with respect to ZOT or zonulin binding or activation of the ZOT receptor, to regulate tight junctional physiology within the methods and compositions of the invention.

VASODILATOR AGENTS AND METHODS

Yet another class of absorption-promoting agents that shows beneficial utility within the coordinate administration and combinatorial formulation methods and compositions of the invention are vasoactive compounds, more specifically vasodilators. These compounds function within the invention to modulate the structure and physiology of the submucosal vasculature, increasing the transport rate of JAM, occludin and claudin peptides, proteins, analogs and mimetics, and other biologically active agents into or through the mucosal epithelium and/or to specific target tissues or compartments (e.g., the systemic circulation or central nervous system.).

Vasodilator agents for use within the invention typically cause submucosal blood vessel relaxation by either a decrease in cytoplasmic calcium, an increase in nitric oxide (NO) or by inhibiting myosin light chain kinase. They are generally divided into 9 classes: calcium antagonists, potassium channel openers, ACE inhibitors, angiotensin-II receptor antagonists, α-adrenergic and imidazole receptor antagonists, β1 -adrenergic agonists, phosphodiesterase inhibitors, eicosanoids and NO donors.

Despite chemical differences, the pharmacokinetic properties of calcium antagonists are similar. Absorption into the systemic circulation is high, and these agents therefore undergo considerable first-pass metabolism by the liver, resulting in

individual variation in pharmacokinetics. Except for the newer drugs of the dihydropyridine type (amlodipine, felodipine, isradipine, nilvadipine, nisoldipine and nitrendipine), the half-life of calcium antagonists is short. Therefore, to maintain an effective drug concentration for many of these may require delivery by multiple dosing, or controlled release formulations, as described elsewhere herein. Treatment with the potassium channel opener minoxidil may also be limited in manner and level of administration due to potential adverse side effects.

ACE inhibitors prevent conversion of angiotensin-I to angiotensin-II, and are most effective when renin production is increased. Since ACE is identical to kininase-II, which inactivates the potent endogenous vasodilator bradykinin, ACE inhibition causes a reduction in bradykinin degradation. ACE inhibitors provide the added advantage of cardioprotective and cardioreparative effects, by preventing and reversing cardiac fibrosis and ventricular hypertrophy in animal models. The predominant elimination pathway of most ACE inhibitors is via renal excretion. Therefore, renal impairment is associated with reduced elimination and a dosage reduction of 25 to 50% is recommended in patients with moderate to severe renal impairment.

With regard to NO donors, these compounds are particularly useful within the invention for their additional effects on mucosal permeability. In addition to the above-noted NO donors, complexes of NO with nucleophiles called NO/nucleophiles, or NONOates, spontaneously and nonenzymatically release NO when dissolved in aqueous solution at physiologic pH (Cornfield et al., J. Lab. Clin. Med., 134(4):419-425, 1999,). In contrast, nitro vasodilators such as nitroglycerin require specific enzyme activity for NO release. NONOates release NO with a defined stoichiometry and at predictable rates ranging from <3 minutes for diethylamine/NO to approximately 20 hours for diethylenetriamine/NO (DETANO).

Within certain methods and compositions of the invention, a selected vasodilator agent is coordinately administered (e.g., systemically or intranasally, simultaneously or in combinatorially effective temporal association) or combinatorially formulated with one or more JAM, occludin and claudin peptides, proteins, analogs and mimetics, and other biologically active agent(s) in an amount effective to enhance the mucosal absorption of the active agent(s) to reach a target tissue or compartment in the subject (e.g., the systemic circulation or CNS).

SELECTIVE TRANSPORT-ENHANCING AGENTS AND METHODS

Within certain aspects of the invention, mucosal delivery of biologically active agents is enhanced by methods and agents that target selective transport mechanisms and promote endo- or transcytocis of macromoloecular drugs. In this regard, the compositions and delivery methods of the invention optionally incorporate a selective transport-enhancing agent that facilitates transport of one or more biologically active agents. These transport-enhancing agents may be employed in a combinatorial formulation or coordinate administration protocol with one or more of the JAM, occludin and claudin peptides, proteins, analogs and mimetics disclosed herein, to coordinately enhance delivery of one or more additional biologically active agent(s) across mucosal transport barriers, to enhance mucosal delivery of the active agent(s) to reach a target tissue or compartment in the subject (e.g., the mucosal epithelium, the systemic circulation or the CNS). Alternatively, the transport-enhancing agents may be employed in a combinatorial formulation or coordinate administration protocol to directly enhance mucosal delivery of one or more of the JAM, occludin and claudin peptides, proteins, analogs and mimetics, with or without enhanced delivery of an additional biologically active agent.

Exemplary selective transport-enhancing agents for use within this aspect of the invention include, but are not limited to, glycosides, sugar-containing molecules, and binding agents such as lectin binding agents, which are known to interact specifically with epithelial transport barrier components (see, e.g., Goldstein et al., Annu. Rev. Cell. Biol. 1:1-39, 1985). For example, specific "bioadhesive" ligands, including various plant and bacterial lectins, which bind to cell surface sugar moieties by receptor-mediated interactions can be employed as carriers or conjugated transport mediators for enhancing mucosal, e.g., nasal delivery of biologically active agents within the invention. Certain bioadhesive ligands for use within the invention will mediate transmission of biological signals to epithelial target cells that trigger selective uptake of the adhesive ligand by specialized cellular transport processes (endocytosis or transcytosis). These transport mediators can therefore be employed as a "carrier system" to stimulate or direct selective uptake of one or more JAM, occludin and claudin peptides, proteins, analogs and mimetics, and other biologically active agent(s) into and/or through mucosal epithelia. These and other selective

transport-enhancing agents significantly enhance mucosal delivery of macromolecular biopharmaceuticals (particularly peptides, proteins, oligonucleotides and polynucleotide vectors) within the invention. To utilize these transport-enhancing agents, general carrier formulation and/or conjugation methods as described elsewhere herein are used to coordinately administer a selective transport enhancer (e.g., a receptor-specific ligand) and a biologically active agent to a mucosal surface, whereby the transport-enhancing agent is effective to trigger or mediate enhanced endo- or transcytosis of the active agent into or across the mucosal epithelium and/or to additional target cell(s), tissue(s) or compartment(s).

Lectins are plant proteins that bind to specific sugars found on the surface of glycoproteins and glycolipids of eukaryotic cells. Concentrated solutions of lectins have a 'mucotractive' effect, and various studies have demonstrated rapid receptor mediated endocytocis (RME) of lectins and lectin conjugates (e.g., concanavalin A conjugated with colloidal gold particles) across mucosal surfaces. Additional studies have reported that the uptake mechanisms for lectins can be utilized for intestinal drug targeting *in vivo*. In certain of these studies, polystyrene nanoparticles (500 nm) were covalently coupled to tomato lectin and reported yielded improved systemic uptake after oral administration to rats.

In addition to plant lectins, microbial adhesion and invasion factors provide a rich source of candidates for use as adhesive/selective transport carriers within the mucosal delivery methods and compositions of the invention (*see, e.g.*, Lehr, Crit. Rev. Therap. Drug Carrier Syst. 11:177-218, 1995; Swann, PA, Pharmaceutical Research 15:826-832, 1998). Two components are necessary for bacterial adherence processes, a bacterial 'adhesin' (adherence or colonization factor) and a receptor on the host cell surface. Bacteria causing mucosal infections need to penetrate the mucus layer before attaching themselves to the epithelial surface. This attachment is usually mediated by bacterial fimbriae or pilus structures, although other cell surface components may also take part in the process. Adherent bacteria colonize mucosal epithelia by multiplication and initiation of a series of biochemical reactions inside the target cell through signal transduction mechanisms (with or without the help of toxins). Associated with these invasive mechanisms, a wide diversity of bioadhesive proteins (e.g., invasin, internalin) originally produced by various bacteria and viruses are known. These allow for extracellular attachment of such microorganisms with an impressive selectivity for host species and even particular target tissues. Signals

transmitted by such receptor-ligand interactions trigger the transport of intact, living microorganisms into, and eventually through, epithelial cells by endo- and transcytotic processes. Such naturally occurring phenomena may be harnessed (e.g., by complexing biologically active agents such as a JAM, occludin, or claudin peptide with an adhesin) according to the teachings herein for enhanced delivery of biologically active compounds into or across mucosal epithelia and/or to other designated target sites of drug action. One advantage of this strategy is that the selective carrier partners thus employed are substrate-specific, leaving the natural barrier function of epithelial tissues intact against other solutes (see, e.g., Lehr, Drug Absorption Enhancement, pp. 325-362, de Boer, Ed., Harwood Academic Publishers, 1994).

Various bacterial and plant toxins that bind epithelial surfaces in a specific, lectin-like manner are also useful within the methods and compositions of the invention. For example, diptheria toxin (DT) enters host cells rapidly by RME. Likewise, the B subunit of the *E. coli* heat labile toxin binds to the brush border of intestinal epithelial cells in a highly specific, lectin-like manner. Uptake of this toxin and transcytosis to the basolateral side of the enterocytes has been reported *in vivo* and *in vitro*. Other researches have expressed the transmembrane domain of diphtheria toxin in E. coli as a maltose-binding fusion protein and coupled it chemically to high-Mw poly-L-lysine. The resulting complex was successfully used to mediate internalization of a reporter gene *in vitro*. In addition to these examples, *Staphylococcus aureus* produces a set of proteins (e.g., staphylococcal enterotoxin A (SEA), SEB, toxic shock syndrome toxin 1 (TSST- 1) which act both as superantigens and toxins. Studies relating to these proteins have reported dose-dependent, facilitated transcytosis of SEB and TSST- I in Caco-2 cells.

Various plant toxins, mostly ribosome-inactivating proteins (RIPs), have been identified that bind to any mammalian cell surface expressing galactose units and are subsequently internalized by RME. Toxins such as nigrin b, α-sarcin, ricin and saporin, viscumin, and modeccin are highly toxic upon oral administration (i.e., are rapidly internalized). Therefore, modified, less toxic subunits of these compound will be useful within the invention to facilitate the uptake of biologically active agents, including JAM, occludin and claudin peptides, proteins, analogs and mimetics.

Viral haemagglutinins comprise another type of transport agent to facilitate mucosal delivery of biologically active agents within the methods and compositions of the invention. The initial step in many viral infections is the binding of surface proteins (haemagglutinins) to mucosal cells. These binding proteins have been identified for most viruses, including rotaviruses, varicella zoster virus, semliki forest virus, adenoviruses, potato leafroll virus, and reovirus. These and other exemplary viral hemagglutinins can be employed in a combinatorial formulation (e.g., a mixture or conjugate formulation) or coordinate administration protocol with one or more of the JAM, occludin and claudin peptides, proteins, analogs and mimetics disclosed herein, to coordinately enhance mucosal delivery of one or more additional biologically active agent(s). Alternatively, viral hemagglutinins can be employed in a combinatorial formulation or coordinate administration protocol to directly enhance mucosal delivery of one or more of the JAM, occludin and claudin peptides, proteins, analogs and mimetics, with or without enhanced delivery of an additional biologically active agent.

A variety of endogenous, selective transport-mediating factors are also available for use within the invention. Mammalian cells have developed an assortment of mechanisms to facilitate the internalization of specific substrates and target these to defined compartments. Collectively, these processes of membrane deformations are termed 'endocytosis' and comprise phagocytosis, pinocytosis, receptor-mediated endocytosis (clathrin-mediated RME), and potocytosis (non-clathrin-mediated RME). RME is a highly specific cellular biologic process by which, as its name implies, various ligands bind to cell surface receptors and are subsequently internalized and trafficked within the cell. In many cells the process of endocytosis is so active that the entire membrane surface is internalized and replaced in less than a half hour.

RME is initiated when specific ligands bind externally oriented membrane receptors. Binding occurs quickly and is followed by membrane invagination until an internal vesicle forms within the cell (the early endosome, "receptosome", or CURL (compartment of uncoupling receptor and ligand). Localized membrane proteins, lipids and extracellular solutes are also internalized during this process. When the ligand binds to its specific receptor, the ligand-receptor complex accumulates in coated pits. Coated pits are areas of the membrane with high concentration of endocellular clathrin subunits. The assembly of clathrin molecules on the coated pit is

believed to aid the invagination process. Specialized coat proteins called adaptins, trap specific membrane receptors that move laterally through the membrane in the coated pit area by binding to a signal sequence (Tyr-X-Arg-Phe (SEQ ID NO: 845), where X = any amino acid) at the endocellular carboxy terminus of the receptor. This process ensures that the correct receptors are concentrated in the coated pit areas and minimizes the amount of extracellular fluid that is taken up in the cell.

Following the internalization process, the clathrin coat is lost through the help of chaperone proteins, and proton pumps lower the endosomal pH to approximately 5.5, which causes dissociation of the receptor-ligand complex. CURL serves as a compartment to segregate the recycling receptor (e.g. transferrin) from receptor involved in transcytosis (e.g. transcoba-lamin). Endosomes may then move randomly or by saltatory motion along the microtubules until they reach the trans-Golgi reticulum where they are believed to fuse with Golgi components or other membranous compartments and convert into tubulovesicular complexes and late endosomes or multivesicular bodies. The fate of the receptor and ligand are determined in these sorting vesicles. Some ligands and receptors are returned to the cell surface where the ligand is released into the extracellular milieu and the receptor is recycled. Alternatively, the ligand is directed to lysosomes for destruction while the receptor is recycled to the cell membrane. The endocytotic recycling pathways of polarized epithelial cells are generally more complex than in non-polarized cells. In these enterocytes a common recycling compartment exists that receives molecules from both apical and basolateral membranes and is able to correctly return them to the appropriate membrane or membrane recycling compartment.

Current understanding of RME receptor structure and related structure-function relationships has been significantly enhanced by the cloning of mRNA sequences coding for endocytotic receptors. Most RME receptors share principal structural features, such as an extracellular ligand binding site, a single hydrophobic transmembrane domain (unless the receptor is expressed as a dimer), and a cytoplasmic tail encoding endocytosis and other functional signals. Two classes of receptors are proposed based on their orientation in the cell membrane; the amino terminus of Type I receptors is located on the extracellular side of the membrane, whereas Type II receptors have this same protein tail in the intracellular milieu.

As noted above, potocytosis, or non-clathrin coated endocytosis, takes place through caveolae, which are uniform omega- or flask-shaped membrane invaginations

50-80 nm in diameter. This process was first described as the internalization mechanism of the vitamin folic acid. Morphological studies have implicated caveolae in i) the transcytosis of macromolecules across endothelial cells; (ii) the uptake of small molecules via potocytosis involving GPI-linked receptor molecules and an unknown anion transport protein; iii) interactions with the actin-based cytoskeleton; and (iv) the compartmentalization of certain signaling molecules involved in signal transduction, including G-protein coupled receptors. Caveolae are characterized by the presence of an integral 22-kDa membrane protein termed VIP21-caveolin, which coats the cytoplasmic surface of the membrane. From a drug delivery standpoint, the advantage of potocytosis pathways over clathrin-coated RME pathways lies in the absence of the pH lowering step, which circumvents the endosomal/lysosomal pathway. This pathway for selective transporter-mediated delivery of biologically active agents is therefore particularly effective for enhanced delivery of pH-sensitive macromolecules.

Exemplary among potocytotic transport carriers mechanisms for use within the invention is the folate carrier system, which mediates transport of the vitamin folic acid (FA) into target cells via specific binding to the folate receptor (FR) (see, e.g., Reddy et al., Crit. Rev. Ther. Drug Car. Syst. 15:587-627, 1998,). The cellular uptake of free folic acid is mediated by the folate receptor and/or the reduced folate carrier. The folate receptor is a glycosylphosphatidylinositol (GPI)-anchored 38 kDa glycoprotein clustered in caveolae mediating cell transport by potocytosis. While the expression of the reduced folate carrier is ubiquitously distributed in eukaryotic cells, the folate receptor is principally overexpressed in human tumors. Two homologous isoforms (α and β) of the receptor have been identified in humans. The α-isoform is found to be frequently overexprssed in epithelial tumors, whereas the β-form is often found in non-epithelial lineage tumors. Consequently, this receptor system has been used in drug-targeting approaches to cancer cells, but also in protein delivery, gene delivery, and targeting of antisense oligonucleotides to a variety of cell types.

Folate-drug conjugates are well suited for use within the mucosal delivery methods of the invention, because they allow penetration of target cells exclusively via FR-mediated endocytosis. When FA is covalently linked, for example, via its γ-carboxyl to a biologically active agent, FR binding affinity (KD~10^{-10}M) is not significantly compromised, and endocytosis proceeds relatively unhindered,

promoting uptake of the attached active agent by the FR-expressing cell. Because FRs are significantly overexpressed on a large fraction of human cancer cells (e.g., ovarian, lung, breast, endometrial, renal, colon, and cancers of myeloid hematopoietic cells), this methodology allows for selective delivery of a wide range of therapeutic as well as diagnostic agents to tumors. Folate-mediated tumor targeting has been exploited to date for delivery of the following classes of molecules and molecular complexes that find use within the invention: (i) protein toxins, (ii) low-molecular-weight chemotherapeutic agents, (iii) radioimaging agents, (iv) MRI contrast agents, (v) radio-therapeutic agents, (vi) liposomes with entrapped drugs, (vii) genes, (viii) antisense oligonucleotides, (ix) ribozymes, and (x) immunotherapeutic agents (see, e.g., Swann, PA, Pharmaceutical Research 15:826-832, 1998). In virtually all cases, *in vitro* studies demonstrate a significant improvement in potency and/or cancer-cell specificity over the nontargeted form of the same pharmaceutical agent.

In addition to the folate receptor pathway, a variety of additional methods to stimulate transcytosis within the invention are directed to the transferrin receptor pathway, and the riboflavin receptor pathway. In one aspect, conjugation of a biologically active agent to riboflavin can effectuate RME-mediated uptake. Yet additional embodiments of the invention utilize vitamin B12 (cobalamin) as a specialized transport protein (e.g., conjugation partner) to facilitate entry of biologically active agents into target cells. Certain studies suggest that this particular system can be employed for the intestinal uptake of luteinizing hormone releasing factor (LHRH)-analogs, granulocyte colony stimulating factor (G-CSF, 18.8 kDa), erythropoietin (29.5 kDa), α-interferon, and the LHRH-antagonist ANTIDE.

Still other embodiments of the invention utilize transferrin as a carrier or stimulant of RME of mucosally delivered biologically active agents. Transferrin, an 80 kDa iron-transporting glycoprotein, is efficiently taken up into cells by RME. Transferrin receptors are found on the surface of most proliferating cells, in elevated numbers on erythroblasts and on many kinds of tumors. According to current knowledge of intestinal iron absorption, transferrin is excreted into the intestinal lumen in the form of apotransferrin and is highly stable to attacks from intestinal peptidases. In most cells, diferric transferrin binds to transferrin receptor (TfR), a dimeric transmembrane glycoprotein of 180 kDa, and the ligand-receptor complex is endocytosed within clathrin-coated vesicles. After acidification of these vesicles, iron

dissociates from the transferrin/TfR complex and enters the cytoplasm, where it is bound by ferritin (Fn). Recent reports suggest that insulin covalently coupled to transferrin, is transported across Caco-2 cell monolayers by RME. Other studies suggest that oral administration of this complex to streptozotocin-induced diabetic mice significantly reduces plasma glucose levels (~ 28%), which is further potentiated by BFA pretreatment. The transcytosis of transferrin (Tf) and transferrin conjugates is reportedly enhanced in the presence of Brefeldin A (BFA), a fungal metabolite. In other studies, BFA treatment has been reported to rapidly increase apical endocytosis of both ricin and HRP in MDCK cells. Thus, BFA and other agents that stimulate receptor-mediated transport can be employed within the methods of the invention as combinatorially formulated (e.g., conjugated) and/or coordinately administered agents to enhance receptor-mediated transport of biologically active agents, including JAM, occludin and claudin peptides, proteins, analogs and mimetics.

Immunoglobulin transport mechanisms provide yet additional endogenous pathways and reagents for incorporation within the mucosal delivery methods and compositions of the invention. Receptor-mediated transcytosis of immunoglobulin G (IgG) across the neonatal small intestine serves to convey passive immunity to many newborn mammals. In rats, IgG in milk selectively binds to neonatal Fc receptors (FcRn) expressed on the surface of the proximal small intestinal enterocytes during the first three weeks after birth. FcRn binds IgG in a pH-dependent manner, with binding occurring at the luminal pH (approx. 6-6.5) of the jejunum and release at the pH of plasma (approx. 7.4). The Fc receptor resembles the major histocompatibility complex (MHC) class I antigens in that it consists of two subunits, a transmembrane glycoprotein (gp50) in association with β2-microglobulin. In mature absorptive cells both subunits are colocalized in each of the membrane compartments that mediate transcytosis of IgG. IgG administered *in situ* apparently causes both subunits to concentrate within endocytic pits of the apical plasma membrane, suggesting that ligand causes redistribution of receptors at this site. These results support a model for transport in which IgG is transferred across the cell as a complex with both subunits.

Within the methods and compositions of the present invention, IgG and other immune system-related carriers (including polyclonal and monoclonal antibodies and various fragments thereof) can be coordinate administered with biologically active agents to provide for targeted delivery, typically by receptor-mediated transport, of

the biologically active agent. For example, the biologically active agent (including JAM, occludin and claudin peptides, proteins, analogs and mimetics) may be covalently linked to the IgG or other immunological active agent or, alternatively, formulated in liposomes or other carrier vehicle which is in turn modified (e.g., coated or covalently linked) to incorporate IgG or other immunological transport enhancer. In certain embodiments, polymeric IgA and/or IgM transport agents are employed, which bind to the polymeric immunoglobulin receptors (pIgRs) of target epithelial cells. Within these methods, expression of pIgR can be enhanced by cytokines.

Within more detailed aspects of the invention, antibodies and other immunological transport agents may be themselves modified for enhanced mucosal delivery, for example, as described in detail elsewhere herein, antibodies may be more effectively administered within the methods and compositions of the invention by charge modifying techniques. In one such aspect, an antibody drug delivery strategy involving antibody cationization is utilized that facilitates both trans-endothelial migration and target cell endocytosis (see, e.g., Pardridge, et al., JPET 286:548-544, 1998). In one such strategy, the pI of the antibody is increased by converting surface carboxyl groups of the protein to extended primary amino groups. These cationized homologous proteins have no measurable tissue toxicity and have minimal immunogenicity. In addition, monoclonal antibodies may be cationized with retention of affinity for the target protein.

Additional selective transport-enhancing agents for use within the invention comprise whole bacteria and viruses, including genetically engineered bacteria and viruses, as well as components of such bacteria and viruses. Aside from conventional gene delivery vectors (e.g., adenovirus), this aspect of the invention includes the use of bacterial ghosts and subunit constructs, e.g., as described by Huter et al., Journal of Controlled Release 61:51-63, 1999. Bacterial ghosts are non-denatured bacterial cell envelopes, for example as produced by the controlled expression of the plasmid-encoded lysis gene *E* of bacteriophage PhiX174 in gram-negative bacteria. Protein E-specific lysis does not cause any physical or chemical denaturation to bacterial surface structures, and bacterial ghosts are therefore useful in development of inactivated whole-cell vaccines. Ghosts produced from *Actinobacillus pleuropneumoniae*, *Pasteurella haemolytica* and *Salmonella* sp. have proved successful in vaccination experiments. Recombinant bacterial ghosts can be created by the expression of foreign genes fused to a membrane-targeting sequence, and thus can carry foreign

therapeutic peptides and proteins anchored in their envelope. The fact that bacterial ghosts preserve a native cell wall, including bioadhesive structures like fimbriae of their living counterparts, makes them suitable for the attachment to specific target tissues such as mucosal surfaces. Bacterial ghosts have been shown to be readily taken up by macrophages, thus adhesion of ghosts to specific tissues can be followed by uptake through phagocytes.

In view of the foregoing, a wide variety of ligands involved in receptor-mediated transport mechanisms are known in the art and can be variously employed within the methods and compositions of the invention (e.g., as conjugate partners or coordinately administered mediators) to enhance receptor-mediated transport of biologically active agents, including JAM, occludin and claudin peptides, proteins, analogs and mimetics, and other biologically active agents disclosed herein. Generally, these ligands include hormones and growth factors, bacterial adhesins and toxins, lectins, metal ions and their carriers, vitamins, immunoglobulins, whole viruses and bacteria or selected components thereof. Exemplary ligands among these classes include, for example, calcitonin, prolactin, epidermal growth factor, glucagon, growth hormone, estrogen, lutenizing hormone, platelet derived growth factor, thyroid stimulating hormone, thyroid hormone, cholera toxin, diptheria toxin, E. coli heat labile toxin, Staphylococcal enterotoxins A and B, ricin, saporin, modeccin, nigrin, sarcin, concanavalin A, transcobalantin, catecholamines, transferrin, folate, riboflavin, vitamin B1, low density lipoprotein, maternal IgO, polymeric IgA, adenovirus, vesicular stomatitis virus, Rous sarcoma virus, *V. cholerae*, *Kiebsiella* strains, *Serratia* strains, parainfluenza virus, respiratory syncytial virus, *Varicella zoster*, and *Enterobacter* strains (see, e.g., Swann, PA, Pharmaceutical Research 15:826-832, 1998).

In certain additional embodiments of the invention, membrane-permeable peptides (e.g., "arginine rich peptides") are employed to facilitate delivery of biologically active agents. While the mechanism of action of these peptides remains to be fully elucidated, they provide useful delivery enhancing adjuncts for use within the mucosal delivery compositions and methods herein. In one example, a basic peptide derived from human immunodeficiency virus (HIV)-1 Tat protein (e.g., residues 48-60) has been reported to translocate effectively through cell membranes and accumulate in the nucleus, a characteristic which can be utilized for the delivery of exogenous proteins into cells. The sequence of Tat (GRKKRRQRRRPPQ) (SEQ

ID NO: 846) comprises a highly basic and hydrophilic peptide, which contains 6 arginine and 2 lysine residues in its 13 amino acid residues. Various other arginine-rich peptides have been identified which have a translocation activity very similar to Tat-(48-60). These include such peptides as the D-amino acid- and arginine-substituted Tat-(48-60), the RNA-binding peptides derived from virus proteins, such as HIV-1 Rev, and flock house virus coat proteins, and the DNA binding segments of leucine zipper proteins, such as cancer-related proteins c-Fos and c-Jun, and the yeast transcription factor GCN4 (see, e.g., Futaki et al., Journal Biological Chemistry 276:5836-5840, 2000,). These peptides reportedly have several arginine residues marking their only identified common structural characteristic, suggesting a common internalization mechanism ubiquitous to arginine-rich peptides, which is not explained by typical endocytosis. Using $(Arg)_n$ (n=4-16) peptides, Futaki et al. teach optimization of arginine residues (n ~ 8) for efficient translocation. Recently, methods have been developed for the delivery of exogenous proteins into living cells with the help of arginine rich membrane-permeable carrier peptides such as HIV-1 Tat- and Antennapedia-(see, Futaki et al., supra, and references cited therein,). By genetically or chemically hybridizing these carrier peptides with biologically active agents as described herein, additional methods and compositions are thus provided within the invention to enhance mucosal delivery.

POLYMERIC DELIVERY VEHICLES AND METHODS

Within certain aspects of the invention, JAM, occludin and claudin peptides, proteins, analogs and mimetics, other biologically active agents disclosed herein, and delivery-enhancing agents as described above, are, individually or combinatorially, incorporated within a mucosally (e.g., nasally) administered formulation that includes a biocompatible polymer functioning as a carrier or base. Such polymer carriers include polymeric powders, matrices or microparticulate delivery vehicles, among other polymer forms. The polymer can be of plant, animal, or synthetic origin. Often the polymer is crosslinked. Additionally, in these delivery systems the biologically active agent (e.g., a JAM, occludin or claudin peptide, protein, analog or mimetic), can be functionalized in a manner where it can be covalently bound to the polymer and rendered inseparable from the polymer by simple washing. In other embodiments, the polymer is chemically modified with an inhibitor of enzymes or other agents which may degrade or inactivate the biologically active agent(s) and/or

delivery enhancing agent(s). In certain formulations, the polymer is a partially or completely water insoluble but water swellable polymer, e.g., a hydrogel. Polymers useful in this aspect of the invention are desirably water interactive and/or hydrophilic in nature to absorb significant quantities of water, and they often form hydrogels when placed in contact with water or aqueous media for a period of time sufficient to reach equilibrium with water. In more detailed embodiments, the polymer is a hydrogel which, when placed in contact with excess water, absorbs at least two times its weight of water at equilibrium when exposed to water at room temperature (see, e.g., U.S. Patent No. 6,004,583).

Drug delivery systems based on biodegradable polymers are preferred in many biomedical applications because such systems are broken down either by hydrolysis or by enzymatic reaction into non-toxic molecules. The rate of degradation is controlled by manipulating the composition of the biodegradable polymer matrix. These types of systems can therefore be employed in certain settings for long-term release of biologically active agents. Biodegradable polymers such as poly(glycolic acid) (PGA), poly-(lactic acid) (PLA), and poly(D,L-lactic-co-glycolic acid) (PLGA), have received considerable attention as possible drug delivery carriers, since the degradation products of these polymers have been found to have low toxicity. During the normal metabolic function of the body these polymers degrade into carbon dioxide and water (Mehta et al, J. Control. Rel. 29:375-384, 1994). These polymers have also exhibited excellent biocompatibility.

For prolonging the biological activity of JAM, occludin and claudin peptides, proteins, analogs and mimetics, and other biologically active agents disclosed herein, as well as optional delivery-enhancing agents, these agents may be incorporated into polymeric matrices, e.g., polyorthoesters, polyanhydrides, or polyesters. This yields sustained activity and release of the active agent(s), e.g., as determined by the degradation of the polymer matrix (Heller, Formulation and Delivery of Proteins and Peptides, pp. 292-305, Cleland et al., Eds., ACS Symposium Series 567, Washington DC, 1994; Tabata et al., Pharm. Res.10:487-496, 1993; and Cohen et al., Pharm. Res.8:713-720, 1991). Although the encapsulation of biotherapeutic molecules inside synthetic polymers may stabilize them during storage and delivery, the largest obstacle of polymer-based release technology is the activity loss of the therapeutic molecules during the formulation processes that often involve heat, sonication or organic solvents (Tabata et al., Pharm. Res.10:487-496, 1993; and Jones et al., Drug

Targeting and Delivery Series, New Delivery Systems for Recombinant Proteins - Practical Issues from Proof of Concept to Clinic, Vol. 4, pp. 57-67, Lee et al., Eds., Harwood Academic Publishers, 1995).

Absorption-promoting polymers contemplated for use within the invention may include derivatives and chemically or physically modified versions of the foregoing types of polymers, in addition to other naturally occurring or synthetic polymers, gums, resins, and other agents, as well as blends of these materials with each other or other polymers, so long as the alterations, modifications or blending do not adversely affect the desired properties, such as water absorption, hydrogel formation, and/or chemical stability for useful application. In more detailed aspects of the invention, polymers such as nylon, acrylan and other normally hydrophobic synthetic polymers may be sufficiently modified by reaction to become water swellable and/or form stable gels in aqueous media.

Suitable polymers for use within the invention should generally be stable alone and in combination with the selected biologically active agent(s) and additional components of a mucosal formulation, and form stable hydrogels in a range of pH conditions from about pH 1 to pH 10. More typically, they should be stable and form polymers under pH conditions ranging from about 3 to 9, without additional protective coatings. However, desired stability properties may be adapted to physiological parameters characteristic of the targeted site of delivery (e.g., nasal mucosa or secondary site of delivery such as the systemic circulation). Therefore, in certain formulations higher or lower stabilities at a particular pH and in a selected chemical or biological environment will be more desirable.

Absorption-promoting polymers of the invention may include polymers from the group of homo- and copolymers based on various combinations of the following vinyl monomers: acrylic and methacrylic acids, acrylamide, methacrylamide, hydroxyethylacrylate or methacrylate, vinylpyrrolidones, as well as polyvinylalcohol and its co- and terpolymers, polyvinylacetate, its co- and terpolymers with the above listed monomers and 2-acrylamido-2-methyl-propanesulfonic acid (AMPS®). Very useful are copolymers of the above listed monomers with copolymerizable functional monomers such as acryl or methacryl amide acrylate or methacrylate esters where the ester groups are derived from straight or branched chain alkyl, aryl having up to four aromatic rings which may contain alkyl substituents of 1 to 6 carbons; steroidal,

sulfates, phosphates or cationic monomers such as N,N-dimethylaminoalkyl(meth)acrylamide, dimethylaminoalkyl(meth)acrylate, (meth)acryloxyalkyltrimethylammonium chloride, (meth)acryloxyalkyldimethylbenzyl ammonium chloride.

Additional absorption-promoting polymers for use within the invention are those classified as dextrans, dextrins, and from the class of materials classified as natural gums and resins, or from the class of natural polymers such as processed collagen, chitin, chitosan, pullalan, zooglan, alginates and modified alginates such as "Kelcoloid" (a polypropylene glycol modified alginate) gellan gums such as "Kelocogel", Xanathan gums such as "Keltrol", estastin, alpha hydroxy butyrate and its copolymers, hyaluronic acid and its derivatives, polylactic and glycolic acids.

A very useful class of polymers applicable within the instant invention are olefinically-unsaturated carboxylic acids containing at least one activated carbon-to-carbon olefinic double bond, and at least one carboxyl group; that is, an acid or functional group readily converted to an acid containing an olefinic double bond which readily functions in polymerization because of its presence in the monomer molecule, either in the alpha-beta position with respect to a carboxyl group, or as part of a terminal methylene grouping. Olefinically-unsaturated acids of this class include such materials as the acrylic acids typified by the acrylic acid itself, alpha-cyano acrylic acid, beta methylacrylic acid (crotonic acid), alpha-phenyl acrylic acid, beta-acryloxy propionic acid, cinnamic acid, p-chloro cinnamic acid, 1-carboxy-4-phenyl butadiene-1,3, itaconic acid, citraconic acid, mesaconic acid, glutaconic acid, aconitic acid, maleic acid, fumaric acid, and tricarboxy ethylene. As used herein, the term "carboxylic acid" includes the polycarboxylic acids and those acid anhydrides, such as maleic anhydride, wherein the anhydride group is formed by the elimination of one molecule of water from two carboxyl groups located on the same carboxylic acid molecule.

Representative acrylates useful as absorption-promoting agents within the invention include methyl acrylate, ethyl acrylate, propyl acrylate, isopropyl acrylate, butyl acrylate, isobutyl acrylate, methyl methacrylate, methyl ethacrylate, ethyl methacrylate, octyl acrylate, heptyl acrylate, octyl methacrylate, isopropyl methacrylate, 2-ethylhexyl methacrylate, nonyl acrylate, hexyl acrylate, n-hexyl methacrylate, and the like. Higher alkyl acrylic esters are decyl acrylate, isodecyl methacrylate, lauryl acrylate, stearyl acrylate, behenyl acrylate and melissyl acrylate

and methacrylate versions thereof. Mixtures of two or three or more long chain acrylic esters may be successfully polymerized with one of the carboxylic monomers. Other comonomers include olefins, including alpha olefins, vinyl ethers, vinyl esters, and mixtures thereof.

Other vinylidene monomers, including the acrylic nitriles, may also be used as absorption-promoting agents within the methods and compositions of the invention to enhance delivery and absorption of one or more JAM, occludin and claudin peptides, proteins, analogs and mimetics, and other biologically active agent(s), including to enhance delivery of the active agent(s) to a target tissue or compartment in the subject (e.g., the systemic circulation or CNS). Useful alpha, beta-olefinically unsaturated nitriles are preferably monoolefinically unsaturated nitriles having from 3 to 10 carbon atoms such as acrylonitrile, methacrylonitrile, and the like. Most preferred are acrylonitrile and methacrylonitrile. Acrylic amides containing from 3 to 35 carbon atoms including monoolefinically unsaturated amides also may be used. Representative amides include acrylamide, methacrylamide, N-t-butyl acrylamide, N-cyclohexyl acrylamide, higher alkyl amides, where the alkyl group on the nitrogen contains from 8 to 32 carbon atoms, acrylic amides including N-alkylol amides of alpha, beta-olefinically unsaturated carboxylic acids including those having from 4 to 10 carbon atoms such as N-methylol acrylamide, N-propanol acrylamide, N-methylol methacrylamide, N-methylol maleimide, N-methylol maleamic acid esters, N-methylol-p-vinyl benzamide, and the like.

Yet additional useful absorption promoting materials are alpha-olefins containing from 2 to 18 carbon atoms, more preferably from 2 to 8 carbon atoms; dienes containing from 4 to 10 carbon atoms; vinyl esters and allyl esters such as vinyl acetate; vinyl aromatics such as styrene, methyl styrene and chloro-styrene; vinyl and allyl ethers and ketones such as vinyl methyl ether and methyl vinyl ketone; chloroacrylates; cyanoalkyl acrylates such as alpha-cyanomethyl acrylate, and the alpha-, beta-, and gamma-cyanopropyl acrylates; alkoxyacrylates such as methoxy ethyl acrylate; haloacrylates as chloroethyl acrylate; vinyl halides and vinyl chloride, vinylidene chloride and the like; divinyls, diacrylates and other polyfunctional monomers such as divinyl ether, diethylene glycol diacrylate, ethylene glycol dimethacrylate, methylene-bis-acrylamide, allylpentaerythritol, and the like; and bis (beta-haloalkyl) alkenyl phosphonates such as bis(beta-chloroethyl) vinyl phosphonate and the like as are known to those skilled in the art. Copolymers

wherein the carboxy containing monomer is a minor constituent, and the other vinylidene monomers present as major components are readily prepared in accordance with the methods disclosed herein.

When hydrogels are employed as absorption promoting agents within the invention, these may be composed of synthetic copolymers from the group of acrylic and methacrylic acids, acrylamide, methacrylamide, hydroxyethylacrylate (HEA) or methacrylate (HEMA), and vinylpyrrolidones which are water interactive and swellable. Specific illustrative examples of useful polymers, especially for the delivery of peptides or proteins, are the following types of polymers: (meth)acrylamide and 0.1 to 99 wt. % (meth)acrylic acid; (meth)acrylamides and 0.1-75 wt % (meth)acryloxyethyl trimethyammonium chloride; (meth)acrylamide and 0.1-75 wt % (meth)acrylamide; acrylic acid and 0.1-75 wt % alkyl(meth)acrylates; (meth)acrylamide and 0.1-75 wt % AMPS.RTM. (trademark of Lubrizol Corp.); (meth)acrylamide and 0 to 30 wt % alkyl(meth)acrylamides and 0.1-75 wt % AMPS.RTM.; (meth)acrylamide and 0.1-99 wt. % HEMA; (metb)acrylamide and 0.1 to 75 wt % HEMA and 0.1 to 99%(meth)acrylic acid; (meth)acrylic acid and 0.1-99 wt % HEMA; 50 mole % vinyl ether and 50 mole % maleic anhydride; (meth)acrylamide and 0.1 to 75 wt % (meth)acryloxyalky dimethyl benzylammonium chloride; (meth)acrylamide and 0.1 to 99 wt % vinyl pyrrolidone; (meth)acrylamide and 50 wt % vinyl pyrrolidone and 0.1-99.9 wt % (meth)acrylic acid; (meth)acrylic acid and 0.1 to 75 wt % AMPS.RTM. and 0.1-75 wt % alkyl(meth)acrylamide. In the above examples, alkyl means C_1 to C_{30}, preferably C_1 to C_{22}, linear and branched and C_4 to C_{16} cyclic; where (meth) is used, it means that the monomers with and without the methyl group are included. Other very useful hydrogel polymers are swellable, but insoluble versions of poly(vinyl pyrrolidone) starch, carboxymethyl cellulose and polyvinyl alcohol.

Additional polymeric hydrogel materials useful within the invention include (poly) hydroxyalkyl (meth)acrylate: anionic and cationic hydrogels: poly(electrolyte) complexes; poly(vinyl alcohols) having a low acetate residual: a swellable mixture of crosslinked agar and crosslinked carboxymethyl cellulose: a swellable composition comprising methyl cellulose mixed with a sparingly crosslinked agar; a water swellable copolymer produced by a dispersion of finely divided copolymer of maleic anhydride with styrene, ethylene, propylene, or isobutylene; a water swellable

polymer of N-vinyl lactams; swellable sodium salts of carboxymethyl cellulose; and the like.

Other gelable, fluid imbibing and retaining polymers useful for forming the hydrophilic hydrogel for mucosal delivery of biologically active agents within the invention include pectin; polysaccharides such as agar, acacia, karaya, tragacenth, algins and guar and their crosslinked versions; acrylic acid polymers, copolymers and salt derivatives, polyacrylamides; water swellable indene maleic anhydride polymers; starch graft copolymers; acrylate type polymers and copolymers with water absorbability of about 2 to 400 times its original weight; diesters of polyglucan; a mixture of crosslinked poly(vinyl alcohol) and poly(N-vinyl-2-pyrrolidone); polyoxybutylene-polyethylene block copolymer gels; carob gum; polyester gels; poly urea gels; polyether gels; polyamide gels; polyimide gels; polypeptide gels; polyamino acid gels; poly cellulosic gels; crosslinked indene-maleic anhydride acrylate polymers; and polysaccharides.

Synthetic hydrogel polymers for use within the invention may be made by an infinite combination of several monomers in several ratios. The hydrogel can be crosslinked and generally possesses the ability to imbibe and absorb fluid and swell or expand to an enlarged equilibrium state. The hydrogel typically swells or expands upon delivery to the nasal mucosal surface, absorbing about 2-5, 5-10, 10-50, up to 50-100 or more times fold its weight of water. The optimum degree of swellability for a given hydrogel will be determined for different biologically active agents depending upon such factors as molecular weight, size, solubility and diffusion characteristics of the active agent carried by or entrapped or encapsulated within the polymer, and the specific spacing and cooperative chain motion associated with each individual polymer.

Hydrophilic polymers useful within the invention are water insoluble but water swellable. Such water swollen polymers as typically referred to as hydrogels or gels. Such gels may be conveniently produced from water soluble polymer by the process of crosslinking the polymers by a suitable crosslinking agent. However, stable hydrogels may also be formed from specific polymers under defined conditions of pH, temperature and/or ionic concentration, according to know methods in the art. Typically the polymers are cross-linked, that is, cross-linked to the extent that the polymers possess good hydrophilic properties, have improved physical integrity (as compared to non cross-linked polymers of the same or similar type) and exhibit

improved ability to retain within the gel network both the biologically active agent of interest and additional compounds for coadministration therewith such as a cytokine or enzyme inhibitor, while retaining the ability to release the active agent(s) at the appropriate location and time.

Generally hydrogel polymers for use within the invention are crosslinked with a difunctional cross-linking in the amount of from 0.01 to 25 weight percent, based on the weight of the monomers forming the copolymer, and more preferably from 0.1 to 20 weight percent and more often from 0. 1 to 15 weight percent of the crosslinking agent. Another useful amount of a crosslinking agent is 0.1 to 10 weight percent. Tri, tetra or higher multifunctional crosslinking agents may also be employed. When such reagents are utilized, lower amounts may be required to attain equivalent crosslinking density, i.e., the degree of crosslinking, or network properties that are sufficient to contain effectively the biologically active agent(s).

The crosslinks can be covalent, ionic or hydrogen bonds with the polymer possessing the ability to swell in the presence of water containing fluids. Such crosslinkers and crosslinking reactions are known to those skilled in the art and in many cases are dependent upon the polymer system. Thus a crosslinked network may be formed by free radical copolymerization of unsaturated monomers. Polymeric hydrogels may also be formed by crosslinking preformed polymers by reacting functional groups found on the polymers such as alcohols, acids, amines with such groups as glyoxal, formaldehyde or glutaraldehyde, bis anhydrides and the like.

The polymers also may be cross-linked with any polyene, e.g. decadiene or trivinyl cyclohexane; acrylamides, such as N,N-methylene-bis (acrylamide); polyfunctional acrylates, such as trimethylol propane triacrylate; or polyfunctional vinylidene monomer containing at least 2 terminal CH.sub.2 < groups, including, for example, divinyl benzene, divinyl naphthlene, allyl acrylates and the like. In certain embodiments, cross-linking monomers for use in preparing the copolymers are polyalkenyl polyethers having more than one alkenyl ether grouping per molecule, which may optionally possess alkenyl groups in which an olefinic double bond is present attached to a terminal methylene grouping (e.g., made by the etherification of a polyhydric alcohol containing at least 2 carbon atoms and at least 2 hydroxyl groups). Compounds of this class may be produced by reacting an alkenyl halide, such as allyl chloride or allyl bromide, with a strongly alkaline aqueous solution of one or more polyhydric alcohols. The product may be a complex mixture of

polyethers with varying numbers of ether groups. Efficiency of the polyether cross-linking agent increases with the number of potentially polymerizable groups on the molecule. Typically, polyethers containing an average of two or more alkenyl ether groupings per molecule are used. Other cross-linking monomers include for example, diallyl esters, dimethallyl ethers, allyl or methallyl acrylates and acrylamides, tetravinyl silane, polyalkenyl methanes, diacrylates, and dimethacrylates, divinyl compounds such as divinyl benzene, polyallyl phosphate, diallyloxy compounds and phosphite esters and the like. Typical agents are allyl pentaerythritol, allyl sucrose, trimethylolpropane triacrylate, 1,6-hexanediol diacrylate, trimethylolpropane diallyl ether, pentaerythritol triacrylate, tetramethylene dimethacrylate, ethylene diacrylate, ethylene dimethacrylate, triethylene glycol dimethacrylate, and the like. Allyl pentaerythritol, trimethylolpropane diallylether and allyl sucrose provide suitable polymers. When the cross-linking agent is present, the polymeric mixtures usually contain between about 0.01 to 20 weight percent, e.g., 1%, 5%, or 10% or more by weight of cross-linking monomer based on the total of carboxylic acid monomer, plus other monomers.

In more detailed aspects of the invention, mucosal delivery of JAM, occludin and claudin peptides, proteins, analogs and mimetics, and other biologically active agents disclosed herein, is enhanced by retaining the active agent(s) in a slow-release or enzymatically or physiologically protective carrier or vehicle, for example a hydrogel that shields the active agent from the action of the degradative enzymes. In certain embodiments, the active agent is bound by chemical means to the carrier or vehicle, to which may also be admixed or bound additional agents such as enzyme inhibitors, cytokines, etc. The active agent may alternately be immobilized through sufficient physical entrapment within the carrier or vehicle, e.g., a polymer matrix.

Polymers such as hydrogels useful within the invention may incorporate functional linked agents such as glycosides chemically incorporated into the polymer for enhancing intranasal bioavailability of active agents formulated therewith. Examples of such glycosides are glucosides, fructosides, galactosides, arabinosides, mannosides and their alkyl substituted derivatives and natural glycosides such as arbutin, phlorizin, amygdalin, digitonin, saponin, and indican. There are several ways in which a typical glycoside may be bound to a polymer. For example, the hydrogen of the hydroxyl groups of a glycoside or other similar carbohydrate may be replaced by the alkyl group from a hydrogel polymer to form an ether. Also, the hydroxyl

groups of the glycosides may be reacted to esterify the carboxyl groups of a polymeric hydrogel to form polymeric esters *in situ*. Another approach is to employ condensation of acetobromoglucose with cholest-5-en-3beta-ol on a copolymer of maleic acid. N-substituted polyacrylamides can be synthesized by the reaction of activated polymers with omega-aminoalkylglycosides: (1) (carbohydrate-spacer)(n)-polyacrylamide, `pseudopolysaccharides`; (2) (carbohydrate spacer)(n)-phosphatidylethanolamine(m)-polyacrylamide, neoglycolipids, derivatives of phosphatidylethanolamine; (3) (carbohydrate-spacer)(n)-biotin(m)-polyacrylamide. These biotinylated derivatives may attach to lectins on the mucosal surface to facilitate absorption of the biologically active agent(s), e.g., a polymer-encapsulated JAM protein or peptide.

Within more detailed aspects of the invention, one or more JAM, occludin and claudin peptides, proteins, analogs and mimetics, and/or other biologically active agents, disclosed herein, optionally including secondary active agents such as protease inhibitor(s), cytokine(s), additional modulator(s) of intercellular junctional physiology, etc., are modified and bound to a polymeric carrier or matrix. For example, this may be accomplished by chemically binding a peptide or protein active agent and other optional agent(s) within a crosslinked polymer network. It is also possible to chemically modify the polymer separately with an interactive agent such as a glycosidal containing molecule. In certain aspects, the biologically active agent(s), and optional secondary active agent(s), may be functionalized, i.e., wherein an appropriate reactive group is identified or is chemically added to the active agent(s). Most often an ethylenic polymerizable group is added, and the functionalized active agent is then copolymerized with monomers and a crosslinking agent using a standard polymerization method such as solution polymerization (usually in water), emulsion, suspension or dispersion polymerization. Often, the functionalizing agent is provided with a high enough concentration of functional or polymerizable groups to insure that several sites on the active agent(s) are functionalized. For example, in a polypeptide comprising 16 amine sites, it is generally desired to functionalize at least 2, 4, 5, 7, up to 8 or more of said sites.

After functionalization, the functionalized active agent(s) is/are mixed with monomers and a crosslinking agent that comprise the reagents from which the polymer of interest is formed. Polymerization is then induced in this medium to create a polymer containing the bound active agent(s). The polymer is then washed

with water or other appropriate solvents and otherwise purified to remove trace unreacted impurities and, if necessary, ground or broken up by physical means such as by stirring, forcing it through a mesh, ultrasonication or other suitable means to a desired particle size. The solvent, usually water, is then removed in such a manner as to not denature or otherwise degrade the active agent(s). One desired method is lyophilization (freeze drying) but other methods are available and may be used (e.g., vacuum drying, air drying, spray drying, etc.).

To introduce polymerizable groups in peptides, proteins and other active agents within the invention, it is possible to react available amino, hydroxyl, thiol and other reactive groups with electrophiles containing unsaturated groups. For example, unsaturated monomers containing N-hydroxy succinimidyl groups, active carbonates such as p-nitrophenyl carbonate, trichlorophenyl carbonates, tresylate, oxycarbonylimidazoles, epoxide, isocyanates and aldehyde, and unsaturated carboxymethyl azides and unsaturated orthopyridyl-disulfide belong to this category of reagents. Illustrative examples of unsaturated reagents are allyl glycidyl ether, allyl chloride, allylbromide, allyl iodide, acryloyl chloride, allyl isocyanate, allylsulfonyl chloride, maleic anhydride, copolymers of maleic anhydride and allyl ether, and the like.

All of the lysine active derivatives, except aldehyde, can generally react with other amino acids such as imidazole groups of histidine and hydroxyl groups of tyrosine and the thiol groups of cystine if the local environment enhances nucleophilicity of these groups. Aldehyde containing functionalizing reagents are specific to lysine. These types of reactions with available groups from lysines, cysteines, tyrosine have been extensively documented in the literature and are known to those skilled in the art.

In the case of biologically active agents that contain amine groups, it is convenient to react such groups with an acyloyl chloride, such as acryloyl chloride, and introduce the polymerizable acrylic group onto the reacted agent. Then during preparation of the polymer, such as during the crosslinking of the copolymer of acrylamide and acrylic acid, the functionalized active agent, through the acrylic groups, is attached to the polymer and becomes bound thereto.

In additional aspects of the invention, biologically active agents, including peptides, proteins, nucleosides, and other molecules which are bioactive *in vivo*, are conjugation-stabilized by covalently bonding one or more active agent(s) to a polymer

incorporating as an integral part thereof both a hydrophilic moiety, e.g., a linear polyalkylene glycol, a lipophilic moiety (see, e.g., U.S. Patent No. 5,681,811,). In one aspect, a biologically active agent is covalently coupled with a polymer comprising (i) a linear polyalkylene glycol moiety and (ii) a lipophilic moiety, wherein the active agent, linear polyalkylene glycol moiety, and the lipophilic moiety are conformationally arranged in relation to one another such that the active therapeutic agent has an enhanced *in vivo* resistance to enzymatic degradation (i.e., relative to its stability under similar conditions in an unconjugated form devoid of the polymer coupled thereto). In another aspect, the conjugation-stabilized formulation has a three-dimensional conformation comprising the biologically active agent covalently coupled with a polysorbate complex comprising (i) a linear polyalkylene glycol moiety and (ii) a lipophilic moiety, wherein the active agent, the linear polyalkylene glycol moiety and the lipophilic moiety are conformationally arranged in relation to one another such that (a) the lipophilic moiety is exteriorly available in the three-dimensional conformation, and (b) the active agent in the composition has an enhanced *in vivo* resistance to enzymatic degradation.

In a further related aspect, a multiligand conjugated complex is provided which comprises a biologically active agent covalently coupled with a triglyceride backbone moiety through a polyalkylene glycol spacer group bonded at a carbon atom of the triglyceride backbone moiety, and at least one fatty acid moiety covalently attached either directly to a carbon atom of the triglyceride backbone moiety or covalently joined through a polyalkylene glycol spacer moiety (see, e.g., U.S. Patent No. 5,681,811). In such a multiligand conjugated therapeutic agent complex, the alpha' and beta carbon atoms of the triglyceride bioactive moiety may have fatty acid moieties attached by covalently bonding either directly thereto, or indirectly covalently bonded thereto through polyalkylene glycol spacer moieties. Alternatively, a fatty acid moiety may be covalently attached either directly or through a polyalkylene glycol spacer moiety to the alpha and alpha' carbons of the triglyceride backbone moiety, with the bioactive therapeutic agent being covalently coupled with the gamma-carbon of the triglyceride backbone moiety, either being directly covalently bonded thereto or indirectly bonded thereto through a polyalkylene spacer moiety. It will be recognized that a wide variety of structural, compositional, and conformational forms are possible for the multiligand conjugated therapeutic agent complex comprising the triglyceride backbone moiety, within the scope of the

invention. It is further noted that in such a multiligand conjugated therapeutic agent complex, the biologically active agent(s) may advantageously be covalently coupled with the triglyceride modified backbone moiety through alkyl spacer groups, or alternatively other acceptable spacer groups, within the scope of the invention. As used in such context, acceptability of the spacer group refers to steric, compositional, and end use application specific acceptability characteristics.

In yet additional aspects of the invention, a conjugation-stabilized complex is provided which comprises a polysorbate complex comprising a polysorbate moiety including a triglyceride backbone having covalently coupled to alpha, alpha' and beta carbon atoms thereof functionalizing groups including (i) a fatty acid group; and (ii) a polyethylene glycol group having a biologically active agent or moiety covalently bonded thereto, e.g., bonded to an appropriate functionality of the polyethylene glycol group (see, e.g., U.S. Patent No. 5,681,811). Such covalent bonding may be either direct, e.g., to a hydroxy terminal functionality of the polyethylene glycol group, or alternatively, the covalent bonding may be indirect, e.g., by reactively capping the hydroxy terminus of the polyethylene glycol group with a terminal carboxy functionality spacer group, so that the resulting capped polyethylene glycol group has a terminal carboxy functionality to which the biologically active agent or moiety may be covalently bonded.

In yet additional aspects of the invention, a stable, aqueously soluble, conjugation-stabilized complex is provided which comprises one or more JAM, occludin and claudin peptides, proteins, analogs and mimetics, and/or other biologically active agent(s)+ disclosed herein covalently coupled to a physiologically compatible polyethylene glycol (PEG) modified glycolipid moiety. In such complex, the biologically active agent(s) may be covalently coupled to the physiologically compatible PEG modified glycolipid moiety by a labile covalent bond at a free amino acid group of the active agent, wherein the labile covalent bond is scissionable *in vivo* by biochemical hydrolysis and/or proteolysis. The physiologically compatible PEG modified glycolipid moiety may advantageously comprise a polysorbate polymer, e.g., a polysorbate polymer comprising fatty acid ester groups selected from the group consisting of monopalmitate, dipalmitate, monolaurate, dilaurate, trilaurate, monoleate, dioleate, trioleate, monostearate, distearate, and tristearate. In such complex, the physiologically compatible PEG modified glycolipid moiety may suitably comprise a polymer selected from the group consisting of polyethylene

glycol ethers of fatty acids, and polyethylene glycol esters of fatty acids, wherein the fatty acids for example comprise a fatty acid selected from the group consisting of lauric, palmitic, oleic, and stearic acids.

BIOADHESIVE DELIVERY VEHICLES AND METHODS

In certain aspects of the invention, the combinatorial formulations and/or coordinate administration methods herein incorporate an effective amount of a nontoxic bioadhesive as an adjunct compound or carrier to enhance mucosal delivery of one or more biologically active agent(s). Bioadhesive agents in this context exhibit general or specific adhesion to one or more components or surfaces of the targeted mucosa. The bioadhesive maintains a desired concentration gradient of the biologically active agent into or across the mucosa to ensure penetration of even large molecules (e.g., peptides and proteins) into or through the mucosal epithelium. Typically, employment of a bioadhesive within the methods and compositions of the invention yields a two- to five- fold, often a five- to ten-fold increase in permeability for peptides and proteins into or through the mucosal epithelium. This enhancement of epithelial permeation often permits effective transmucosal delivery of large macromolecules, for example to the basal portion of the nasal epithelium or into the adjacent extracellular compartments or the systemic circulation or CNS.

This enhanced delivery provides for greatly improved effectiveness of delivery of bioactive peptides, proteins and other macromolecular therapeutic species. These results will depend in part on the hydrophilicity of the compound, whereby greater penetration will be achieved with hydrophilic species compared to water insoluble compounds. In addition to these effects, employment of bioadhesives to enhance drug persistence at the mucosal surface can elicit a reservoir mechanism for protracted drug delivery, whereby compounds not only penetrate across the mucosal tissue but also back-diffuse toward the mucosal surface once the material at the surface is depleted.

A variety of suitable bioadhesives are disclosed in the art for oral administration (*see, e.g.*, U.S. Patent Nos. 3,972,995; 4,259,314; 4,680,323; 4,740,365; 4,573,996; 4,292,299; 4,715,369; 4,876,092; 4,855,142; 4,250,163; 4,226,848; 4,948,580; U.S. Pat. Reissue 33,093; and Robinson, 18 Proc. Intern. Symp.

Control. Rel. Bioact. Mater. 75 (1991)), which find use within the novel methods and compositions of the invention. The potential of various bioadhesive polymers as a mucosal, e.g., nasal, delivery platform within the methods and compositions of the invention can be readily assessed by determining their ability to retain and release a specific biologically active agent, e.g., a JAM, occludin, or claudin peptide or protein, as well as by their capacity to interact with the mucosal surfaces following incorporation of the active agent therein. In addition, well known methods will be applied to determine the biocompatibility of selected polymers with the tissue at the site of mucosal administration. One aspect of polymer biocompatibility is the potential effect for the polymer to induce a cytokine response. In certain circumstances, implanted polymers have been shown to induce the release of inflammatory cytokines from adhering cells, such as monocytes and macrophages. Similar potential adverse reactions of mucosal epithelial cells in contact with candidate bioadhesive polymers will be determined using routine *in vitro* and *in vivo* assays. Since epithelial cells have the ability to secrete a number of cytokines, the induction of cytokine responses in epithelial cells will often provide an adequate measure of biocompatibility of a selected polymer delivery platform.

When the target mucosa is covered by mucus (i.e., in the absence of mucolytic or mucus-clearing treatment), it can serve as a connecting link to the underlying mucosal epithelium. Therefore, the term "bioadhesive" as used herein also covers mucoadhesive compounds useful for enhancing mucosal delivery of biologically active agents within the invention. However, adhesive contact to mucosal tissue mediated through adhesion to a mucus gel layer may be limited by incomplete or transient attachment between the mucus layer and the underlying tissue, particularly at nasal surfaces where rapid mucus clearance occurs. In this regard, mucin glycoproteins are continuously secreted and, immediately after their release from cells or glands, form a viscoelastic gel. The luminal surface of the adherent gel layer, however, is continuously eroded by mechanical, enzymatic and/or ciliary action. Where such activities are more prominent, or where longer adhesion times are desired, the coordinate administration methods and combinatorial formulation methods of the invention may further incorporate mucolytic and/or ciliostatic methods or agents as disclosed herein above.

Bioadhesive and other delivery enhancing agents within the methods and compositions of the invention can improve the effectiveness of a treatment by helping

maintain the drug concentration between effective and toxic levels, by inhibiting dilution of the drug away from the delivery point, and improving targeting and localization of the drug. In this context, bioadhesion increases the intimacy and duration of contact between a drug-containing polymer and the mucosal surface. The combined effects of this enhanced, direct drug absorption, and the decrease in excretion rate that results from reduced diffusion and improved localization, significantly enhances bioavailability of the drug and allows for a smaller dosage and less frequent administration.

Typically, mucoadhesive polymers for use within the invention are natural or synthetic macromolecules which adhere to wet mucosal tissue surfaces by complex, but non-specific, mechanisms. In addition to these mucoadhesive polymers, the invention also provides methods and compositions incorporating bioadhesives that adhere directly to a cell surface, rather than to mucus, by means of specific, including receptor-mediated, interactions. One example of bioadhesives that function in this specific manner is the group of compounds known as lectins. These are glycoproteins with an ability to specifically recognize and bind to sugar molecules, e.g. glycoproteins or glycolipids, which form part of intranasal epithelial cell membranes and can be considered as "lectin receptors".

In various embodiments, the coordinate administration methods of the invention optionally incorporate bioadhesive materials that yield prolonged residence time at the mucosal surface. Alternatively, the bioadhesive material may otherwise facilitate mucosal absorption of the biologically active agent, e.g., by facilitating localization of the active agent to a selected target site of activity (e.g., bloodstream or CNS). In additional aspects, adjunct delivery or combinatorial formulation of bioadhesive agents within the methods and compositions of the invention intensify contact of the biologically active agent with the target mucosa, including by increasing epithelial permeability, (e.g., to effectively increase the drug concentration gradient). In further alternate embodiments, bioadhesives and other polymers disclosed herein serve to inhibit proteolytic or other enzymes that might degrade the biologically active agent. For a review of different approaches to bioadhesion that are useful within the coordinate administration, multi-processing and/or combinatorial formulation methods and compositions of the invention, see, e.g., Lehr C. M., Eur J. Drug Metab. Pharmacokinetics 21(2):139-148, 1996.

In certain aspects of the invention, bioadhesive materials for enhancing intranasal delivery of biologically active agents comprise a matrix of a hydrophilic, e.g., water soluble or swellable, polymer or a mixture of polymers that can adhere to a wet mucous surface. These adhesives may be formulated as ointments, hydrogels (see above) thin films, and other application forms. Often, these adhesives have the biologically active agent mixed therewith to effectuate slow release or local delivery of the active agent. Some are formulated with additional ingredients to facilitate penetration of the active agent through the nasal mucosa, e.g., into the circulatory system of the individual.

Various polymers, both natural and synthetic ones, show significant binding to mucus and/or mucosal epithelial surfaces under physiological conditions. The strength of this interaction can readily be measured by mechanical peel or shear tests. A variety of suitable test methods and instruments to serve such purposes are known in the art (see, e.g., Gu et al., <u>Crit. Rev. Ther. Drug Carrier Syst.</u> 5:21-67, 1988; Duchene et al., <u>Drug Dev. Ind. Pharm.</u> 14:283-318, 1988). When applied to a humid mucosal surface, many dry materials will spontaneously adhere, at least slightly. After such an initial contact, some hydrophilic materials start to attract water by adsorption, swelling or capillary forces, and if this water is absorbed from the underlying substrate or from the polymer-tissue interface, the adhesion may be sufficient to achieve the goal of enhancing mucosal absorption of biologically active agents (see, e.g., Al-Dujaili et al., <u>Int. J. Pharm.</u> 34:75-79, 1986; Marvola et al., <u>J. Pharm. Sci.</u> 72:1034-1036, 1983; Marvola et al., <u>J. Pharm. Sci.</u> 71:975-977, 1982; and Swisher et al., <u>Int. J. Pharm.</u> 22:219, 1984; Chen, et al., <u>Adhesion in Biological Systems</u>, p. 172, Manly, Ed., Academic Press, London, 1970). Such 'adhesion by hydration' can be quite strong, but formulations adapted to employ this mechanism must account for swelling which continues as the dosage transforms into a hydrated mucilage. This is projected for many hydrocolloids useful within the invention, especially some cellulose-derivatives, which are generally non-adhesive when applied in pre-hydrated state. Nevertheless, bioadhesive drug delivery systems for mucosal administration are effective within the invention when such materials are applied in the form of a dry polymeric powder, microsphere, or film-type delivery form.

Other polymers adhere to mucosal surfaces not only when applied in dry, but also in fully hydrated state, and in the presence of excess amounts of water. The selection of a mucoadhesive thus requires due consideration of the conditions,

physiological as well as physico-chemical, under which the contact to the tissue will be formed and maintained. In particular, the amount of water or humidity usually present at the intended site of adhesion, and the prevailing pH, are known to largely affect the mucoadhesive binding strength of different polymers.

Several polymeric bioadhesive drug delivery systems have been fabricated and studied in the past 20 years, not always with success. A variety of such carriers are, however, currently used in clinical applications involving dental, orthopedic, ophthalmological, and surgical uses. For example, acrylic-based hydrogels have been used extensively for bioadhesive devices. Acrylic-based hydrogels are well-suited for bioadhesion due to their flexibility and nonabrasive characteristics in the partially swollen state which reduce damage-causing attrition to the tissues in contact [Park et al., J. Control. Release 2:47-57 (1985)]. Furthermore, their high permeability in the swollen state allows unreacted monomer, un-crosslinked polymer chains, and the initiator to be washed out of the matrix after polymerization, which is an important feature for selection of bioadhesive materials for use within the invention. Acrylic-based polymer devices exhibit very high adhesive bond strength, as determined by various known methods (Park et al., J. Control. Release 2:47-57, 1985; Park et al., Pharm. Res. 4:457-464, 1987; and Ch'ng et al., J. Pharm. Sci. 74:399-405, 1985).

For controlled mucosal delivery of peptide and protein drugs, the methods and compositions of the invention optionally include the use of carriers, e.g., polymeric delivery vehicles, that function in part to shield the biologically active agent from proteolytic breakdown, while at the same time providing for enhanced penetration of the peptide or protein into or through the nasal mucosa. In this context, bioadhesive polymers have demonstrated considerable potential for enhancing oral drug delivery. As an example, the bioavailability of 9-desglycinamide, 8-arginine vasopressin (DGAVP) intraduodenally administered to rats together with a 1% (w/v) saline dispersion of the mucoadhesive poly(acrylic acid) derivative polycarbophil, was 3-5-fold increased compared to an aqueous solution of the peptide drug without this polymer (Lehr et al., J. Pharm. Pharmacol.44:402-407, 1992). In this study, the drug was not bound to or otherwise integrally associated with the mucoadhesive polymer in the formulation, which would therefore not be expected to yield enhanced peptide absorption via prolonged residence time or intensified contact to the mucosal surface. Thus, certain bioadhesive polymers for use within the invention will directly enhance

the permeability of the epithelial absorption barrier in part by protecting the active agent, e.g., peptide or protein, from enzymatic degradation.

Recent studies have shown that mucoadhesive polymers of the poly(acrylic acid)-type are potent inhibitors of some intestinal proteases (Lueßen et al., Pharm. Res. 12:1293-1298, 1995; Lueßen et al., J. Control. Rel. 29:329-338, 1994; and Bai et al., J. Pharm. Sci. 84:1291-1294; 1995). The mechanism of enzyme inhibition is explained by the strong affinity of this class of polymers for divalent cations, such as calcium or zinc, which are essential cofactors of metallo-proteinases, such as trypsin and chymotrypsin. Depriving the proteases of their cofactors by poly(acrylic acid) was reported to induce irreversible structural changes of the enzyme proteins which were accompanied by a loss of enzyme activity. At the same time, other mucoadhesive polymers (e.g., some cellulose derivatives and chitosan) may not inhibit proteolytic enzymes under certain conditions. In contrast to other enzyme inhibitors contemplated for use within the invention (e.g. aprotinin, bestatin), which are relatively small molecules, the trans-nasal absorption of inhibitory polymers is likely to be minimal in light of the size of these molecules, and thereby eliminate possible adverse side effects. Thus, mucoadhesive polymers, particularly of the poly(acrylic acid)-type, may serve both as an absorption-promoting adhesive and enzyme-protective agent to enhance controlled delivery of peptide and protein drugs, especially when safety concerns are considered.

In addition to protecting against enzymatic degradation, bioadhesives and other polymeric or non-polymeric absorption-promoting agents for use within the invention may directly increase mucosal permeability to biologically active agents. To facilitate the transport of large and hydrophilic molecules, such as peptides and proteins, across the nasal epithelial barrier, mucoadhesive polymers and other agents have been postulated to yield enhanced permeation effects beyond what is accounted for by prolonged premucosal residence time of the delivery system. For example, nasal administration of insulin to non-primate mammals in the presence of mucoadhesive starch microspheres yielded a steeply enhanced early absorption peak, followed by a continuous decline (Bjork et al., Int. J. Pharm. 47:233-238, 1988; Farraj et al., J. Control. Rel. 13:253-262, 1990). The time course of drug plasma concentrations reportedly suggested that the bioadhesive microspheres caused an acute, but transient increase of insulin permeability across the nasal mucosa. In other studies using *in vitro* cultured epithelial cell monolayers (Bjork et al., J. Drug

Targeting, 1995), it was reported that dry, swellable materials such as starch microspheres induce reversible focal dilations of the tight junctions, allowing for enhanced drug transport along the paracellular route. According to this adhesion-dehydration theory, the hydrophilic polymer, applied as a dry powder, absorbs water from the mucosal tissue in such a way that the epithelial cells are dehydrated and shrink until the normally tight intercellular junctions between the cells become physically separated. Because this effect is of relatively short duration and appears to be completely reversible, it provides yet another useful tool for incorporation within the coordinate administration, multi-processing and/or combinatorial formulation methods and compositions of the invention.

Other mucoadhesive polymers for use within the invention, for example chitosan, reportedly enhance the permeability of certain mucosal epithelia even when they are applied as an aqueous solution or gel (Lehr et al., Int. J. Pharmaceut. 78:43-48, 1992; Illum et al., Pharm. Res.11:1186-1189, 1994; Artursson et al., Pharm. Res. 11:1358-1361, 1994; and Borchard, et al., J. Control. Release 39:131-138, 1996,). In one study, absorption of the peptide drugs insulin and calcitonin, and the hydrophilic compound phenol red, from an aqueous gel base of poly(acrylic acid) was reported after rectal, vaginal and nasal administration (Morimoto et al., Int. J. Pharm. 14:149-157, 1983; and Morimoto et al., J. Pharmacobiodyn. 10:85-91, 1987). Another mucoadhesive polymer reported to directly affect epithelial permeability is hyaluronic acid. In particular, hyaluronic acid gel formulation reportedly enhanced nasal absorption of vasopressin and some of its analogues (Morimoto et al., Pharm. Res.8:471-474, 1991,). Hyaluronic acid was also reported to increase the absorption of insulin from the conjunctiva in diabetic dogs (Nomura, et al., J. Pharm. Pharmacol. 46:768-770, 1994). Ester derivatives of hyaluronic acid in the form of lyophilized microspheres were described as a nasal delivery system for insulin (Illum et al., J. Contr. Rel. 29:133-141, 1994).

A particularly useful bioadhesive agent within the coordinate administration, and/or combinatorial formulation methods and compositions of the invention is chitosan, as well as its analogs and derivatives. Chitosan is a non-toxic, biocompatible and biodegradable polymer that is widely used for pharmaceutical and medical applications because of its favorable properties of low toxicity and good biocompatibility (Yomota, Pharm. Tech. Japan 10:557-564, 1994). It is a natural polyaminosaccharide prepared from chitin by N-deacetylation with alkali. A wide

variety of biomedical uses for chitosan have been reported over the last two decades, based for example on its reported wound healing, antimicrobial and hemostatic properties (Kas, J. Microencapsulation 14:689-711, 1997). Chitosan has also been used as a pharmaceutical excipient in conventional dosage forms as well as in novel applications involving bioadhesion and transmucosal drug transport (Illum, Pharm. Res. 15:1326-1331, 1998; and Olsen et al., Chitin and Chitosan-sources, Chemistry, Biochemistry, Physical Properties and Applications, pp. 813-828, Skjak-Braek et al., Eds., Elsevier, London, 1989). Furthermore, chitosan has been reported to promote absorption of small polar molecules and peptide and protein drugs through nasal mucosa in animal models and human volunteers (Illum et al., Pharm. Res.11:1186-1189, 1994). Other studies have shown an enhancing effect on penetration of compounds across the intestinal mucosa and cultured Caco-2 cells (Schipper et al., Pharm. res. 14:23-29, 1997; and Kotze et al., Int. J. Pharm. 159:243-253, 1997,). Chitosan has also been proposed as a bioadhesive polymer for use in oral mucosal drug delivery (Miyazaki et al., Biol. Pharm. Bull. 17:745-747, 1994; Ikinci et al., Advances in Chitin Science, Vol. 4, Peter et al., Eds., University of Potsdam, in press; Senel, et al., Int. J. Pharm. 193:197-203, 2000; Needleman, et al., J. Clin. Periodontol.24:394-400, 1997). Initial studies showed that chitosan has an extended retention time on the oral mucosa (Needleman et al., J. Clin. Periodontol. 25:74-82, 1998) and with its antimicrobial properties and biocompatibility is an excellent candidate for the treatment of oral mucositis. More recently, Senel et al., Biomaterials 21:2067-2071, (2000) reported that chitosan provides an effective gel carrier for delivery of the bioactive peptide, transforming growth factor-β (TGF-β).

As used within the methods and compositions of the invention, chitosan increases the retention of JAM, occludin and claudin peptides, proteins, analogs and mimetics, and other biologically active agents disclosed herein at a mucosal site of application. This is may be mediated in part by a positive charge characteristic of chitosan, which may influence epithelial permeability even after physical removal of chitosan from the surface (Schipper et al., Pharm. Res. 14:23-29, 1997). Another mechanism of action of chitosan for improving transport of biologically active agents across mucosal membranes may be attributed to transient opening of the tight junctions in the cell membrane to allow polar compounds to penetrate (Illum et al., Pharm. Res.11:1186-1189, 1994; Lueben et al., J. Control. Rel. 29:329-338, 1994). Chitosan may also increase the thermodynamic activity of other absorption-promoting

agents used in certain formulations of the invention, resulting in enhanced penetration. Lastly, as chitosan has been reported to disrupt lipid micelles in the intestine (Muzzarelli et al., EUCHIS'99, Third International Conference of the European Chitin Society, Abstract Book , ORAD-PS-059, Potsdam, Germany, 1999), its absorption-promoting effects may be due in part to its interference with the lipid organization in the mucosal epithelium.

As with other bioadhesive gels provided herein, the use of chitosan can reduce the frequency of application and the amount of biologically active agent administered while yielding an effective delivery amount or dose. This mode of administration can also improve patient compliance and acceptance. The occlusion and lubrication of chitosan and other bioadhesive gels is expected to reduce the discomfort of inflammatory, allergic and ulcerative conditions of the nasal mucosa. In addition, chitosan acts non-specifically on certain deleterious microorganisms, including fungi (Knapczyk, Chitin World, pp. 504-511, Karnicki et al., Eds., Wirtschaftverlag NW, Germany, 1994), and may also beneficially stimulate cell proliferation and tissue organization by acting as an inductive primer to repair and physiologically rebuild damaged tissue (Muzzarelli et al. (Biomaterials 10:598-603, 1989).

As further provided herein, the methods and compositions of the invention will optionally include a novel chitosan derivative or chemically modified form of chitosan. One such novel derivative for use within the invention is denoted as a β-[1→4]-2-guanidino-2-deoxy-D-glucose polymer (poly-GuD). Chitosan is the N-deacetylated product of chitin, a naturally occurring polymer that has been used extensively to prepare microspheres for oral and intra-nasal formulations. The chitosan polymer has also been proposed as a soluble carrier for parenteral drug delivery. Within one aspect of the invention, o-methylisourea is used to convert a chitosan amine to its guanidinium moiety. The guanidinium compound is prepared, for example, by the reaction between equi-normal solutions of chitosan and o-methylisourea at pH above 8.0.

The guanidinium product is -[14]-guanidino-2-deoxy-D-glucose polymer. It is abbreviated as Poly-GuD in this context (Monomer F.W. of Amine in Chitosan = 161; Monomer F.W. of Guanidinium in Poly-GuD = 203).

One exemplary Poly-GuD preparation method for use within the invention involves the following protocol.

<u>Solutions</u>:

Preparation of 0.5% Acetic Acid Solution (0.088N):

Pipette 2.5 mL glacial acetic acid into a 500 mL volumetric flask, dilute to volume with purified water.

Preparation of 2N NaOH Solution:

Transfer about 20 g NaOH pellets into a beaker with about 150 mL of purified water. Dissolve and cool to room temperature. Transfer the solution into a 250-mL volumetric flask, dilute to volume with purified water.

Preparation of O-methylisourea Sulfate (0.4N urea group equivalent):

Transfer about 493 mg of O-methylisourea sulfate into a 10-mL volumetric flask, dissolve and dilute to volume with purified water.

The pH of the solution is 4.2

Preparation of Barium Chloride Solution (0.2M):

Transfer about 2.086 g of Barium chloride into a 50-mL volumetric flask, dissolve and dilute to volume with purified water.

Preparation of Chitosan Solution (0.06N amine equivalent):

Transfer about 100 mg Chitosan into a 50 mL beaker, add 10 mL 0.5% Acetic Acid (0.088 N). Stir to dissolve completely.

The pH of the solution is about 4.5

Preparation of O-methylisourea Chloride Solution (0.2N urea group equivalent):

Pipette 5.0 mL of O-methylisourea sulfate solution (0.4 N urea group equivalent) and 5 mL of 0.2M Barium chloride solution into a beaker. A precipitate is formed. Continue to mix the solution for additional 5 minutes. Filter the solution through 0.45m filter and discard the precipitate. The concentration of O-methylisourea chloride in the supernatant solution is 0.2 N urea group equivalent.

The pH of the solution is 4.2.

<u>Procedure</u>:

Add 1.5 mL of 2 N NaOH to 10 mL of the chitosan solution (0.06N amine equivalent) prepared as described in Section 2.5. Adjust the pH of the solution with 2N NaOH to about 8.2 to 8.4. Stir the solution for additional 10 minutes. Add 3.0 mL O-methylisourea chloride solution (0.2N urea group equivalent) prepared as described above. Stir the solution overnight.

Adjust the pH of solution to 5.5 with 0.5% Acetic Acid (0.088N).

Dilute the solution to a final volume of 25 mL using purified water.

The Poly-GuD concentration in the solution is 5 mg/mL, equivalent to 0.025 N (guanidium group).

Additional compounds classified as bioadhesive agents for use within the present invention act by mediating specific interactions, typically classified as "receptor-ligand interactions" between complementary structures of the bioadhesive compound and a component of the mucosal epithelial surface. Many natural examples illustrate this form of specific binding bioadhesion, as exemplified by lectin-sugar interactions. Lectins are (glyco)proteins of non-immune origin which bind to polysaccharides or glycoconjugates. By virtue of this binding potential, lectins may bind or agglutinate cells (Goldstein et al., Nature 285:66, 1980). Lectins are commonly of plant or bacterial origin, but are also produced by higher animals (so-called 'endogenous or 'reverse' lectins), including mammals (Sharon et al., Lectins, Chapman and Hall, London, 1989; and Pasztai et al., Lectins. Biomedical Perspectives, Taylor & Francis, London, 1995).

Several plant lectins have been investigated as possible pharmaceutical absorption-promoting agents. One plant lectin, Phaseolus vulgaris hemagglutinin (PHA), exhibits high oral bioavailability of more than 10% after feeding to rats (Pusztai et al., Biochem. Soc. Trans. 17:81-82, 1988,). However, PHA has been reported to cause digestive disorders following oral administration, and these side effects must be determined to be minimized by any nasal therapeutic application herein. In contrast, tomato (*Lycopersicon esculeutum*) lectin (TL) appears safe for various modes of administration. This glycoprotein (approximately 70 kDa) resists digestion and binds to rat intestinal villi without inducing any deleterious effects (Kilpatrick, et al., FEBS Lett. 185:5-10, 1985; Woodley et al., Int. J. Pharm. 110:127-136, 1994; and Int. J. Pharm. 107:223-230, 1994). However, GI transit of this radiolabeled lectin after intragastric administration to rats was not delayed compared to controls, and other studies showed that TL has a strong cross-reactivity with gastrointestinal mucus glycoproteins (Lehr, et al., Pharm. Res. 9:547-553, 1992). Thus, in spite of its favorable safety profile, the use of TL as a gastrointestinal bioadhesive, even though its action is "specific" (i.e., receptor-mediated) is limited by non-specific interactions with mucus—promoting rapid clearance.

Therefore, the invention provides for coordinate administration or combinatorial formulation of non-toxic lectins identified or obtained by modification of existing lectins which have a high specific affinity for mucosal, e.g., nasal epithelial, cells, but low cross reactivity with mucus. In this regard, detailed teachings regarding lectin structure-activity relationships will allow selection of non-toxic, strongly bioadhesive candidates to produce optimized lectins for therapeutic purposes, which undertaking will be further facilitated by methods of recombinant gene technology (see, e.g., Lehr et al., Lectins: Biomedical Perspectives, pp. 117-140, Pustai et al., Eds., Taylor and Francis, London, 1995,). In additional embodiments of the invention, mucolytic agents and/or ciliostatic agents are coordinately administered or combinatorially formulated with a biologically active agent and a lectin or other specific binding bioadhesive—in order to counter the effects of non-specific binding of the bioadhesive to mucosal mucus.

In addition to the use of lectins, certain antibodies or amino acid sequences exhibit high affinity binding to complementary elements on cell and mucosal surfaces. Thus, for example, various adhesive amino acids sequences such as Arg-Gly-Asp and others, if attached to a carrier matrix, will promote adhesion by binding with specific cell surface glycoproteins. In other embodiments, adhesive ligand components are integrated in a carrier or delivery vehicle that selectively adheres to a particular cell type, or diseased target tissue. For example, certain diseases cause changes in cell surface glycoproteins. These distinct structural alterations can be readily targeted by complementary amino acid sequences bound to a drug delivery vehicle within the invention. In exemplary aspects, well known cancer-specific markers (e.g., CEA, HER2) may be targeted by complementary antibodies or peptides for specific drug targeting to diseased cells.

In summary, the foregoing bioadhesive agents are useful in the combinatorial formulations and coordinate administration methods of the instant invention, which optionally incorporate an effective amount and form of a bioadhesive agent to prolong persistence or otherwise increase mucosal absorption of one or more JAM, occludin and claudin peptides, proteins, analogs and mimetics, and other biologically active agents. The bioadhesive agents may be coordinately administered as adjunct compounds or as additives within the combinatorial formulations of the invention. In certain embodiments, the bioadhesive agent acts as a 'pharmaceutical glue', whereas

in other embodiments adjunct delivery or combinatorial formulation of the bioadhesive agent serves to intensify contact of the biologically active agent with the nasal mucosa, in some cases by promoting specific receptor-ligand interactions with epithelial cell "receptors", and in others by increasing epithelial permeability to significantly increase the drug concentration gradient measured at a target site of delivery (e.g., the CNS or in the systemic circulation). Yet additional bioadhesive agents for use within the invention act as enzyme (e.g., protease) inhibitors to enhance the stability of mucosally administered biotherapeutic agents delivered coordinately or in a combinatorial formulation with the bioadhesive agent.

<u>LIPOSOMES AND MICELLAR DELIVERY VEHICLES</u>

The coordinate administration methods and combinatorial formulations of the instant invention optionally incorporate effective lipid or fatty acid based carriers, processing agents, or delivery vehicles, to provide improved formulations for mucosal delivery of JAM, occludin and claudin peptides, proteins, analogs and mimetics, and other biologically active agents. For example, a variety of formulations and methods are provided for mucosal delivery which compris+e one or more of these active agents, such as a peptide or protein, admixed or encapsulated by, or coordinately administered with, a liposome, mixed micellar carrier, or emulsion, to enhance chemical and physical stability and increase the half life of the biologically active agents (e.g., by reducing susceptibility to proteolysis, chemical modification and/or denaturation) upon mucosal delivery.

Within certain aspects of the invention, specialized delivery systems for biologically active agents comprise small lipid vesicles known as liposomes (see, e.g., Chonn et al., <u>Curr. Opin. Biotechnol.</u> 6:698-708, 1995; Lasic, <u>Trends Biotechnol.</u> 16:307-321, 1998; and Gregoriadis, <u>Trends Biotechnol.</u> 13:527-537, 1995). These are typically made from natural, biodegradable, non-toxic, and non-immunogenic lipid molecules, and can efficiently entrap or bind drug molecules, including peptides and proteins, into, or onto, their membranes. The attractiveness of liposomes as a peptide and protein delivery system within the invention is increased by the fact that the encapsulated proteins can remain in their preferred aqueous environment within the vesicles, while the liposomal membrane protects them against proteolysis and other

destabilizing factors. Even though not all liposome preparation methods known are feasible in the encapsulation of peptides and proteins due to their unique physical and chemical properties, several methods allow the encapsulation of these macromolecules without substantial deactivation (see, e.g., Weiner, Immunomethods 4:201-209, 1994).

A variety of methods are available for preparing liposomes for use within the invention (e.g., as described in Szoka et al., Ann. Rev. Biophys. Bioeng. 9:467, 1980; and U.S. Pat. Nos. 4,235,871, 4,501,728, and 4,837,028). For use with liposome delivery, the biologically active agent is typically entrapped within the liposome, or lipid vesicle, or is bound to the outside of the vesicle. Several strategies have been devised to increase the effectiveness of liposome-mediated delivery by targeting liposomes to specific tissues and specific cell types. Liposome formulations, including those containing a cationic lipid, have been shown to be safe and well tolerated in human patients (Treat et al., J. Natl. Cancer Instit. 82:1706-1710, 1990).

Like liposomes, unsaturated long chain fatty acids, which also have enhancing activity for mucosal absorption, can form closed vesicles with bilayer-like structures (so called "ufasomes"). These can be formed, for example, using oleic acid to entrap biologically active peptides and proteins for mucosal, e.g., intranasal, delivery within the invention.

Other delivery systems for use within the invention combine the use of polymers and liposomes to ally the advantageous properties of both vehicles. Exemplifying this type of hybrid delivery system, liposomes containing the model protein horseradish peroxidase (HRP) have been effectively encapsulated inside the natural polymer fibrin (Henschen et al., Blood Coagulation, pp. 171-241, Zwaal, et al., Eds., Elsevier, Amsterdam, 1986,). Because of its biocompatibility and biodegradability, fibrin is a useful polymer matrix for drug delivery systems in this context (see, e.g., Senderoff, et al., J. Parenter. Sci. Technol. 45:2-6, 1991; and Jackson, Nat. Med.2:637-638, 1996). In addition, release of biotherapeutic compounds from this delivery system is controllable through the use of covalent crosslinking and the addition of antifibrinolytic agents to the fibrin polymer (Uchino et al., Fibrinolysis 5:93-98, 1991).

More simplified delivery systems for use within the invention include the use of cationic lipids as delivery vehicles or carriers, which can be effectively employed to provide an electrostatic interaction between the lipid carrier and such charged

biologically active agents as proteins and polyanionic nucleic acids (see, e.g., Hope et al., Molecular Membrane Biology 15:1-14, 1998). This allows efficient packaging of the drugs into a form suitable for mucosal administration and/or subsequent delivery to systemic compartments. These and related systems are particularly well suited for delivery of polymeric nucleic acids, e.g., in the form of gene constructs, antisense oligonucleotides and ribozymes. These drugs are large, usually negatively charged molecules with molecular weights on the order of 10_6 for a gene to 10_3 for an oligonucleotide. The targets for these drugs are intracellular, but their physical properties prevent them from crossing cell membranes by passive diffusion as with conventional drugs. Furthermore, unprotected DNA is degraded within minutes by nucleases present in normal plasma. To avoid inactivation by endogenous nucleases, antisense oligonucleotides and ribozymes can be chemically modified to be enzyme resistant by a variety of known methods, but plasmid DNA must ordinarily be protected by encapsulation in viral or non-viral envelopes, or condensation into a tightly packed particulate form by polycations such as proteins or cationic lipid vesicles. More recently, small unilamellar vesicles (SUVs) composed of a cationic lipid and dioleoylphosphatidylethanolamine (DOPE) have been successfully employed as vehicles for polynucleic acids, such as plasmid DNA, to form particles capable of transportation of the active polynucleotide across plasma membranes into the cytoplasm of a broad spectrum of cells. This process (referred to as lipofection or cytofection) is now widely employed as a means of introducing plasmid constructs into cells to study the effects of transient gene expression. Exemplary delivery vehicles of this type for use within the invention include cationic lipids (e.g., N-(2,3-(dioleyloxy)propyl)-N,N,N-trimethyl am-monium chloride (DOTMA)), quarternary ammonium salts (e.g., N ,N-dioleyl-N, N-dimethylammonium chloride (DODAC)), cationic derivatives of cholesterol (e.g., 3β(N-(N',N-dimethylaminoethane-carbamoyl-cholesterol (DC-chol)), and lipids characterized by multivalent headgroups (e.g., dioctadecyldimethylammonium chloride (DOGS), commercially available as Transfectam®).

Additional delivery vehicles for use within the invention include long and medium chain fatty acids, as well as surfactant mixed micelles with fatty acids (see, e.g., Muranishi, Crit. Rev. Ther. Drug Carrier Syst. 7:1-33, 1990,). Most naturally occurring lipids in the form of esters have important implications with regard to their

own transport across mucosal surfaces. Free fatty acids and their monoglycerides which have polar groups attached have been demonstrated in the form of mixed micelles to act on the intestinal barrier as penetration enhancers. This discovery of barrier modifying function of free fatty acids (carboxylic acids with a chain length varying from 12 to 20 carbon atoms) and their polar derivatives has stimulated extensive research on the application of these agents as mucosal absorption enhancers.

For use within the methods of the invention, long chain fatty acids, especially fusogenic lipids (unsaturated fatty acids and monoglycerides such as oleic acid, linoleic acid, linoleic acid, monoolein, etc.) provide useful carriers to enhance mucosal delivery of JAM, occludin and claudin peptides, proteins, analogs and mimetics, and other biologically active agents disclosed herein. Medium chain fatty acids (C6 to C12) and monoglycerides have also been shown to have enhancing activity in intestinal drug absorption and can be adapted for use within the mocosal delivery formulations and methods of the invention. In addition, sodium salts of medium and long chain fatty acids are effective delivery vehicles and absorption-enhancing agents for mucosal delivery of biologically active agents within the invention. Thus, fatty acids can be employed in soluble forms of sodium salts or by the addition of non-toxic surfactants, e.g., polyoxyethylated hydrogenated castor oil, sodium taurocholate, etc. Mixed micelles of naturally occurring unsaturated long chain fatty acids (oleic acid or linoleic acid) and their monoglycerides with bile salts have been shown to exhibit absorption-enhancing abilities, which are basically harmless to the intestinal mucosa (see, e.g., Muranishi, Pharm. Res. 2:108-118, 1985; and Crit. Rev. Ther. drug carrier Syst. 7:1-33, 1990). Other fatty acid and mixed micellar preparations that are useful within the invention include, but are not limited to, Na caprylate (C8), Na caprate (C10), Na laurate (C12) or Na oleate (C18), optionally combined with bile salts, such as glycocholate and taurocholate.

PEGYLATION

Additional methods and compositions provided within the invention involve chemical modification of biologically active peptides and proteins by covalent attachment of polymeric materials, for example dextrans, polyvinyl pyrrolidones, glycopeptides, polyethylene glycol and polyamino acids. The resulting conjugated

peptides and proteins retain their biological activities and solubility for mucosal administration. In alternate embodiments, JAM, occludin and claudin peptides, proteins, analogs and mimetics, and other biologically active peptides and proteins, are conjugated to polyalkylene oxide polymers, particularly polyethylene glycols (PEG) (see, e.g., U.S. Pat. No. 4,179,337). Numerous reports in the literature describe the potential advantages of pegylated peptides and proteins, which often exhibit increased resistance to proteolytic degradation, increased plasma half-life, increased solubility and decreased antigenicity and immunogenicity (Nucci, et al., Advanced Drug Deliver Reviews 6:133-155, 1991; Lu et al., Int. J. Peptide Protein Res. 43:127-138, 1994). A number of proteins, including L-asparaginase, strepto-kinase, insulin, interleukin-2, adenosine deamidase, L-asparaginase, interferon alpha 2b, superoxide dismutase, streptokinase, tissue plasminogen activator (tPA), urokinase, uricase, hemoglobin, TGF-beta, EGF, and other growth factors, have been conjugated to PEG and evaluated for their altered biochemical properties as therapeutics (see, e.g., Ho, et al., Drug Metabolism and Disposition 14:349-352, 1986; Abuchowski et al., Prep. Biochem. 9:205-211, 1979; and Rajagopaian et al., J. Clin. Invest. 75:413-419, 1985, Nucci et al., Adv. Drug Delivery Rev. 4:133-151, 1991). Although the *in vitro* biological activities of pegylated proteins may be decreased, this loss in activity is usually offset by the increased *in vivo* half-life in the bloodstream (Nucci, et al., Advanced Drug Deliver Reviews 6:133-155, 1991,). Accordingly, these and other polymer-coupled peptides and proteins exhibit enhanced properties, such as extended half-life and reduced immunogenicity, when administered mucoally according to the methods and formulations herein.

Several procedures have been reported for the attachment of PEG to proteins and peptides and their subsequent purification (Abuchowski et al., J. Biol. Chem. 252:3582-3586,1977; Beauchamp et al., Anal. Biochem. 131:25-33, 1983). In addition, Lu et al., Int. J. Peptide Protein Res. 43:127-138, 1994 () describe various technical considerations and compare PEGylation procedures for proteins versus peptides (see also, Katre et al., Proc. Natl. Acad. Sci. USA 84:1487-1491, 1987; Becker et al., Makromol. Chem. Rapid Commun. 3:217-223, 1982; Mutter et al., Makromol. Chem. Rapid Commun. 13:151-157, 1992; Merrifield, R.B., J. Am. Chem. Soc. 85:2149-2154, 1993; Lu et al., Peptide Res. 6:142-146, 1993; Lee et al., Bioconjugate Chem. 10:973-981, 1999, Nucci et al., Adv. Drug Deliv. Rev. 6:133-151, 1991; Francis et al., J. Drug Targeting 3:321-340, 1996; Zalipsky, S.,

Bioconjugate Chem. 6:150-165, 1995; Clark et al., J. Biol. Chem. 271:21969-21977, 1996; Pettit et al., J. Biol. Chem. 272:2312-2318, 1997; Delgado et al., Br. J. Cancer 73:175-182, 1996; Benhar et al., Bioconjugate Chem. 5:321-326, 1994; Benhar et al., J. Biol. Chem. 269:13398-13404, 1994; Wang et al., Cancer Res. 53:4588-4594, 1993; Kinstler et al., Pharm. Res. 13:996-1002, 1996, Filpula et al., Exp. Opin. Ther. Patents 9:231-245, 1999; Pelegrin et al., Hum. Gene Ther. 9:2165-2175, 1998).

Following these and other teachings in the art, the conjugation of biologically active peptides and proteins for with polyethyleneglycol polymers, is readily undertaken, with the expected result of prolonging circulating life and/or reducing immunogenicity while maintaining an acceptable level of activity of the PEGylated active agent. Amine-reactive PEG polymers for use within the invention include SC-PEG with molecular masses of 2000, 5000, 10000, 12000, and 20 000; U-PEG-10000; NHS-PEG-3400-biotin; T-PEG-5000; T-PEG-12000; and TPC-PEG-5000. Chemical conjugation chemistries for these polymers have been published (see, e.g., Zalipsky, S., Bioconjugate Chem. 6:150-165, 1995; Greenwald et al., Bioconjugate Chem. 7:638-641, 1996; Martinez et al., Macromol. Chem. Phys. 198:2489-2498, 1997; Hermanson, G. T. , Bioconjugate Techniques, pp. 605-618, 1996; Whitlow et al., Protein Eng. 6:989-995, 1993; Habeeb, A. F. S. A. , Anal. Biochem. 14:328-336, 1966; Zalipsky et al., Poly(ethyleneglycol) Chemistry and Biological Applications, pp. 318-341, 1997; Harlow et al., Antibodies: a Laboratory Manual, pp. 553-612, Cold Spring harbor Laboratory, Plainview, NY, 1988; Milenic et al, Cancer Res. 51:6363-6371, 1991; Friguet et al., J. Immunol. Methods 77:305-319, 1985,). While phosphate buffers are commonly employed in these protocols, the choice of borate buffers may beneficially influence the PEGylation reaction rates and resulting products.

PEGylation of biologically active peptides and proteins may be achieved by modification of carboxyl sites (e.g., aspartic acid or glutamic acid groups in addition to the carboxyl terminus). The utility of PEG-hydrazide in selective modification of carbodiimide-activated protein carboxyl groups under acidic conditions has been described (Zalipsky, S., Bioconjugate Chem. 6:150-165, 1995; Zalipsky et al., Poly(ethyleneglycol) Chemistry and Biological Applications, pp. 318-341, American Chemical Society, Washington, DC, 1997). Alternatively, bifunctional PEG modification of biologically active peptides and proteins can be employed. In some procedures, charged amino acid residues, including lysine, aspartic acid, and glutamic

acid, have a marked tendency to be solvent accessible on protein surfaces. Conjugation to carboxylic acid groups of proteins is a less frequently explored approach for production of protein bioconjugates. However, the hydrazide/EDC chemistry described by Zalipsky and colleagues (Zalipsky, S., Bioconjugate Chem. 6:150-165, 1995; Zalipsky et al., Poly(ethyleneglycol) Chemistry and Biological Applications, pp. 318-341, American Chemical Society, Washington, DC, 1997) offers a practical method of linking PEG polymers to protein carboxylic sites. For example, this alternate conjugation chemistry has been shown to be superior to amine linkages for PEGylation of brain-derived neurotrophic factor (BDNF) while retaining biological activity (Wu et al., Proc. Natl. Acad. Sci. U.S.A. 96:254-259, 1999). Maeda and colleagues have also found carboxyl-targeted PEGylation to be the preferred approach for bilirubin oxidase conjugations (Maeda et al., Poly(ethylene glycol) Chemistry. Biotechnical and Biomedical Applications, J. M. Harris, Ed., pp. 153-169, Plenum Press, New York, 1992).

Often, PEGylation of peptides and proteins for use within the invention involves activating PEG with a functional group that will react with lysine residues on the surface of the peptide or protein. Within certain alternate aspects of the invention, biologically active peptides and proteins are modified by PEGylation of other residues such as His, Trp, Cys, Asp, Glu, etc., without substantial loss of activity. If PEG modification of a selected peptide or protein proceeds to completion, the activity of the peptide or protein is often diminished. Therefore, PEG modification procedures herein are generally limited to partial PEGylation of the peptide or protein, resulting in less than about 50%, more commonly less than about 25%, loss of activity, while providing for substantially increased half-life (e.g., serum half life) and a substantially decreased effective dose requirement of the PEGylated active agent.

An unavoidable result of partial PEG modification is the production of a heterogenous mixture of PEGylated peptide or protein having a statistical distribution of the number of PEG groups bound per molecule. In addition, the usage of lysine residues within the peptide or protein is random. These two factors result in the production of a heterogeneous mixture of PEGylated proteins which differ in both the number and position of the PEG groups attached. For instance, when adenosine deaminase is optimally modified there is a loss of 50% activity when the protein has about 14 PEG per protein, with a broad distribution of the actual number of PEG moieties per individual protein and a broad distribution of the position of the actual

lysine residues used. Such mixtures of diversely modified proteins are not optimally suited for pharmaceutical use. At the same time, purification and isolation of a class of PEGylated proteins (e.g., proteins containing the same number of PEG moieties) or a single type of PEGylated protein (e.g., proteins containing both the same number of moieties and having the PEG moieties at the same position) involves time-consuming and expensive procedures which result in an overall reduction in the yield of the specific PEGylated peptide or protein of interest.

Within certain alternate aspects of the invention, biologically active peptides and proteins are modified by PEGylation methods that employ activated PEG reagents that react with thio groups of the protein, resulting in covalent attachment of PEG to a cysteine residue, which residue may be inserted in place of a naturally-occurring lysine residue of the protein. As described, for example, in U.S. Pat. No. 5,166,322, specific variants of IL-3 have been successfully produced which have a cysteine residue introduced at selected sites within the naturally occurring amino acid sequence. Sulfhydryl reactive compounds (e.g. activated polyethylene glycol) are then attached to these cysteines by reaction with the IL-3 variant. Additionally, U.S. Pat. No. 5,206,344 describes specific IL-2 variants which contain a cysteine residue introduced at a selected sites within the naturally-occurring amino acid sequence. The IL-2 variant is subsequently reacted with an activated polyethylene glycol reagent to attach this moiety to a cysteine residue.

Yet additional methods employed within the invention for generating PEGylated peptides and proteins do not require extensive knowledge of protein structure-function (e.g., mapping amino acid residues essential for biological activity). Exemplifying these methods, U.S. Patent No. 5,766,897 describes methods for production and characterization of cysteine-PEGylated proteins suitable for therapeutic applications. These are produced by attaching a polyethylene glycol to a cysteine residue within the protein. To obtain the desired result of a stable, biologically active compound the PEG is attached in a specific manner, often to a cysteine residue present at or near a site that is normally glycosylated. Typically, the specific amino acid modified by glycosylation (e.g., asparagine in N-linked glycosylation or serine or threonine in O-linked glycosylation) is replaced by a cysteine residue, which is subsequently chemically modified by attachment of PEG. It may be useful for employment of this method to generation cysteine-containing mutants of selected biologically active peptides and proteins, which can be readily

accomplished by, for example, site-directed mutagenesis using methods well known in the art (see, e.g., Kunkel, in Nucleic Acids and Molecular Biology, Eckstein, F. Lilley, D. M. J., eds., Springer-Verlag, Berling and Heidelberg, vol. 2, p. 124, 1988). In addition, if the active peptide or protein is one member of a family of structurally related proteins, glycosylation sites for any other member can be matched to an amino acid on the protein of interest, and that amino acid changed to cysteine for attachment of the polyethylene glycol. Alternatively, if a crystal structure has been determined for the protein of interest or a related protein, surface residues away from the active site or binding site can be changed to cysteine for the attachment of polyethylene glycol.

These strategies for identifying useful PEG attachment sites for use within the invention are advantageous in that they are readily implemented without extensive knowledge of protein structure-function details. Moreover, these strategies also take advantage of the fact that the presence and location of glycosylation residues are often related, as a natural evolutionary consequence, to increased stability and serum half-life of the subject peptide or protein. Replacement of these glycosylation residues by cysteine, followed by cysteine-specific PEGylation, commonly yields modified peptides and proteins that retain substantial biological activity while exhibiting significantly increased stability.

If a higher degree of PEG modification is required, and/or if the peptide or protein to be chemically modified is not normally glycosylated, other solvent accessible residues can be changed to cysteine, and the resultant protein subjected to PEGylation. Appropriate residues can easily be determined by those skilled in the art. For instance, if a three-dimensional structure is available for the protein of interest, or a related protein, solvent accessible amino acids are easily identified. Also, charged amino acids such as Lys, Arg, Asp and Glu are almost exclusively found on the surface of proteins. Substitution of one, two or many of these residues with cysteine will provide additional sites for PEG attachment. In addition, amino acid sequences in the native protein that are recognized by antibodies are usually on the surface of the protein. These and other methods for determining solvent accessible amino acids are well known to those skilled in the art.

Modification of peptides and proteins with PEG can also be used to generate multimeric complexes of proteins, fragments, and/or peptides that have increased biological stability and/or potency. These multimeric peptides and proteins of the

invention, e.g., dimers or tetramers of a JAM, occludin, or claudin peptide or protein, may be produced synthetically according to well known methods. Alternatively, other biologically active peptides and proteins may be produced in this manner that are naturally occurring dimeric or multimeric proteins. For example, dimeric peptides and proteins useful within the invention may be produced by reacting the peptide or protein with $(Maleimido)_2$ -PEG, a reagent composed of PEG having two protein-reactive moieties. In the case of cysteine-pegylated peptides and proteins, the degree of multimeric cross-linking can be controlled by the number of cysteines either present and/or engineered into the peptide or protein, and by the concentration of reagents, e.g., $(Maleimido)_2$ PEG, used in the reaction mixture.

It is further contemplated to attach other groups to thio groups of cysteines present in biologically active peptides and proteins for use within the invention. For example, the peptide or protein may be biotinylated by attaching biotin to a thio group of a cysteine residue. Examples of cysteine-PEGylated proteins of the invention, as well as proteins having a group other than PEG covalently attached via a cysteine residue according to the invention, are as follows:

OTHER STABILIZING MODIFICATIONS OF ACTIVE AGENTS

In addition to PEGylation, biologically active agents such as peptides and proteins for use within the invention can be modified to enhance circulating half-life by shielding the active agent via conjugation to other known protecting or stabilizing compounds, for example by the creation of fusion proteins with an active peptide, protein, analog or mimetic linked to one or more carrier proteins, such as one or more immunoglobulin chains (see, e.g., U.S. Patent Nos. 5,750,375; 5,843,725; 5,567,584 and 6,018,026). These modifications will decrease the degradation, sequestration or clearance of the active agent and result in a longer half-life in a physiological environment (e.g., in the circulatory system, or at a mucosal surface). The active agents modified by these and other stabilizing conjugations methods are therefore useful with enhanced efficacy within the methods of the invention. In particular, the active agents thus modified maintain activity for greater periods at a target site of delivery or action compared to the unmodified active agent. Even when the active

agent is thus modified, it retains substantial biological activity in comparison to a biological activity of the unmodified compound.

Thus, in certain aspects of the invention, JAM, occludin and claudin peptides, proteins, analogs and mimetics, and other biologically active agents, including other active peptides and proteins, for mucosal administration according to the methods of the invention are modified for enhanced activity, e.g., to increase circulating half-life, by shielding the active agent through conjugation to other known protecting or stabilizing compounds, or by the creation of fusion proteins with the peptide, protein, analog or mimetic linked to one or more carrier proteins, such as one or more immunoglobulin chains (see, e.g., U.S. Patent Nos. 5,750,375; 5,843,725; 5,567,584; and 6,018,026). These modifications will decrease the degradation, sequestration or clearance of the active peptide or protein and result in a longer half-life in a physiological environment (e.g., at the nasal mucosal surface or in the systemic circulation). The active peptides and proteins thus modified exhibit enhanced efficacy within the compositions and methods of the invention, for example by increased or temporally extended activity at a target site of delivery or action compared to the unmodified peptide, protein, analog or mimetic.

In other aspects of the invention, peptide and protein therapeutic compounds are conjugated for enhanced stability with relatively low molecular weight compounds, such as aminolethicin, fatty acids, vitamin B_{12}, and glycosides (see, e.g., Igarishi et al., Proc. Int. Symp. Control. Rel. Bioact. Materials, 17, 366, (1990). Additional exemplary modified peptides and proteins for use within the compositions and methods of the invention will be beneficially modified for *in vivo* use by:

(a) chemical or recombinant DNA methods to link mammalian signal peptides (see, e.g., Lin et al., J. Biol. Chem. 270:14255, 1995) or bacterial peptides (see, e.g., Joliot et al., Proc. Natl. Acad. Sci. USA 88:1864, 1991) to the active peptide or protein, which serves to direct the active peptide or protein across cytoplasmic and organellar membranes and/or traffic the active peptide or protein to the a desired intracellular compartment (e.g., the endoplasmic reticulum (ER) of antigen presenting cells (APCs), such as dendritic cells for enhanced CTL induction);

(b) addition of a biotin residue to the active peptide or protein which serves to direct the active conjugate across cell membranes by virtue of its ability to bind specifically (i.e., with a binding affinity greater than about 10^6, 10^7, 10^8, 10^9, or

10^{10} M^{-1}) to a translocator present on the surface of cells (Chen et al., Analytical Biochem. 227:168, 1995);

(c) addition at either or both the amino- and carboxy-terminal ends of the active peptide or protein of a blocking agent in order to increase stability *in vivo*. This can be useful in situations in which the termini of the active peptide or protein tend to be degraded by proteases prior to cellular uptake or during intracellular trafficking. Such blocking agents can include, without limitation, additional related or unrelated peptide sequences that can be attached to the amino and/or carboxy terminal residues of the therapeutic polypeptide or peptide to be administered. This can be done either chemically during the synthesis of the peptide or by recombinant DNA technology. Blocking agents such as pyroglutamic acid or other molecules known to those skilled in the art can also be attached to the amino and/or carboxy terminal residues, or the amino group at the amino terminus or carboxyl group at the carboxy terminus can be replaced with a different moiety.

Biologically active agents modified by PEGylation and other stabilizing methods for use within the methods and compositions of the invention will preferably retain at least 25%, more preferably at least 50%, even more preferably between about 50% to 75%, most preferably 100% of the biological activity associated with the unmodified active agent, e.g., a native peptide or protein. Typically, the modified active agent, e.g., a conjugated peptide or protein, has a half-life ($t_{1/2}$), for example in serum following mucosal delivery, which is enhanced relative to the half-life of the unmodified active agent from which it was derived. In certain aspects, the half-life of a modified active agent (e.g., JAM, occludin and claudin peptides, proteins, analogs and mimetics, and other biologically active peptides and proteins disclosed herein) for use within the invention is enhanced by at least 1.5-fold to 2-fold, often by about 2-fold to 3-fold, in other cases by about 5-fold to 10-fold, and up to 100-fold or more relative to the half-life of the unmodified active agent.

PRODRUG MODIFICATIONS

Yet another processing and formulation strategy useful within the invention is that of prodrug modification. By transiently (i.e., bioreversibly) derivatizing such groups as carboxyl, hydroxyl, and amino groups in small organic molecules, the undesirable physicochemical characteristics (e.g., charge, hydrogen bonding potential, etc. that diminish mucosal penetration) of these molecules can be "masked" without

permanently altering the pharmacological properties of the molecule. Bioreversible prodrug derivatives of therapeutic small molecule drugs have been shown to improve the physicochemical (e.g., solubility, lipophilicity) properties of numerous exemplary therapeutics, particularly those that contain hydroxyl and carboxylic acid groups.

One approach to making prodrugs of amine-containing active agents, such as the peptides and proteins of the invention, is through the acylation of the amino group. Optionally, the use of acyloxyalkoxycarbamate derivatives of amines as prodrugs has been discussed. 3-(2'-hydroxy-4',6'-dimethylphenyl)-3,3-dimethylpropionic acid has been employed to prepare linear, esterase-, phosphatase-, and dehydrogenase-sensitive prodrugs of amines (Amsberry et al., Pharm. Res. 8:455-461, 1991; Wolfe et al., J. Org. Chem. 57:6138, 1992). These systems have been shown to degrade through a two-step mechanism, with the first step being the slow, rate-determining enzyme-catalyzed (esterase, phosphatase, or dehydrogenase) step, and the second step being a rapid ($t_{1/2}$ =100 sec., pH 7.4, 37°C) chemical step (Amsberry et al., J. Org. Chem. 55:5867-5877, 1990). Interestingly, the phosphatase-sensitive system has recently been employed to prepare a very water-soluble (greater than 10 mg/ml) prodrug of TAXOL which shows significant antitumor activity *in vivo*. These and other prodrug modification systems and resultant therapeutic agents are useful within the methods and compositions of the invention.

For the purpose of preparing prodrugs of peptides that are useful within the invention, U.S. Patent No. 5,672,584 further describes the preparation and use of cyclic prodrugs of biologically active peptides and peptide nucleic acids (PNAs). To produce these cyclic prodrugs, the N-terminal amino group and the C-terminal carboxyl group of a biologically active peptide or PNA is linked via a linker, or the C-terminal carboxyl group of the peptide is linked to a side chain amino group or a side chain hydroxyl group via a linker, or the N-terminal amino group of said peptide is linked to a side chain carboxyl group via a linker, or a side chain carboxyl group of said peptide is linked to a side chain amino group or a side chain hydroxyl group via a linker. Useful linkers in this context include 3-(2'-hydroxy-4',6'-dimethyl phenyl)-3,3-dimethyl propionic acid linkers and its derivatives, and acyloxyalkoxy derivatives. The incorporated disclosure provides methods useful for the production and characterization of cyclic prodrugs synthesized from linear peptides, e.g., opioid peptides that exhibit advantageous physicochemical features (e.g., reduced size,

intramolecular hydrogen bond, and amphophilic characteristics) for enhanced cell membrane permeability and metabolic stability. These methods for peptide prodrug modification are also useful to prepare modified peptide therapeutic derivatives for use within the methods and compositions of the invention.

PURIFICATION AND PREPARATION

Biologically active agents for mucosal administration according to the invention, for example JAM, occludin and claudin peptides, proteins, analogs and mimetics, and other biologically active agents disclosed herein, are generally provided for direct administration to subjects in a substantially purified form. The term "substantially purified" as used herein, is intended to refer to a peptide, protein, nucleic acid or other compound that is isolated in whole or in part from naturally associated proteins and other contaminants, wherein the peptide, protein, nucleic acid or other active compound is purified to a measurable degree relative to its naturally-occurring state, e.g., relative to its purity within a cell extract.

Generally, substantially purified peptides, proteins and other active compounds for use within the invention comprise more than 80% of all macromolecular species present in a preparation prior to admixture or formulation of the peptide, protein or other active agent with a pharmaceutical carrier, excipient, buffer, absorption enhancing agent, stabilizer, preservative, adjuvant or other co-ingredient in a complete pharmaceutical formulation for therapeutic administration. More typically, the peptide or other active agent is purified to represent greater than 90%, often greater than 95% of all macromolecular species present in a purified preparation prior to admixture with other formulation ingredients. In other cases, the purified preparation of active agent may be essentially homogeneous, wherein other macromolecular species are not detectable by conventional techniques.

See, for example, R. Scopes, Protein Purification: Principles and Practice, Springer-Verlag: New York, 1982.

Techniques for making substitution mutations at predetermined sites in DNA include for example M13 mutagenesis. Manipulation of DNA sequences to produce substitutional, insertional, or deletional variants are conveniently described elsewhere,

such as in Sambrook et al. (Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y., 1989).

A variety of procaryotic expression systems can be used to express biologically active peptides and proteins for use within the invention. Examples include *E. coli, Bacillus, Streptomyces*, and the like. Detection of the expressed peptide is achieved by methods such as radioimmunoassay, Western blotting techniques or immunoprecipitation. For expression in eukaryotes, host cells for use in practicing the invention include mammalian, avian, plant, insect, and fungal cells. Fungal cells, including species of yeast (e.g., *Saccharomyces* spp., *Schizosaccharomyces* spp.) or filamentous fungi (e.g., *Aspergillus* spp., *Neurospora* spp.) may be used as host cells within the present invention. Strains of the yeast *Saccharomyces cerevisiae* can be used.

FORMULATION AND ADMINISTRATION

Mucosal delivery formulations of the present invention comprise the biologically active agent to be administered (e.g., one or more of the JAM, occludin and claudin peptides, proteins, analogs and mimetics, and other biologically active agents disclosed herein), typically combined together with one or more pharmaceutically acceptable carriers and, optionally, other therapeutic ingredients. The carrier(s) must be "pharmaceutically acceptable" in the sense of being compatible with the other ingredients of the formulation and not eliciting an unacceptable deleterious effect in the subject. Such carriers are described herein above or are otherwise well known to those skilled in the art of pharmacology. Desirably, the formulation should not include substances such as enzymes or oxidizing agents with which the biologically active agent to be administered is known to be incompatible. The formulations may be prepared by any of the methods well known in the art of pharmacy.

Within the compositions and methods of the invention, the JAM, occludin and claudin peptides, proteins, analogs and mimetics, and other biologically active agents disclosed herein may be administered to subjects by a variety of mucosal administration modes, including by oral, rectal, vaginal, intranasal, intrapulmonary, or transdermal delivery, or by topical delivery to the eyes, ears, skin or other mucosal surfaces. Optionally, JAM, occludin and claudin peptides, proteins, analogs and

mimetics, and other biologically active agents disclosed herein can be coordinately or adjunctively administered by non-mucosal routes, including by intramuscular, subcutaneous, intravenous, intra-atrial, intra-articular, intraperitoneal, or parenteral routes. In other alternative embodiments, the biologically active agent(s) can be administered *ex vivo* by direct exposure to cells, tissues or organs originating from a mammalian subject, for example as a component of an *ex vivo* tissue or organ treatment formulation that contains the biologically active agent in a suitable, liquid or solid carrier.

Compositions according to the present invention are often administered in an aqueous solution as a nasal or pulmonary spray and may be dispensed in spray form by a variety of methods known to those skilled in the art. Preferred systems for dispensing liquids as a nasal spray are disclosed in U.S. Pat. No. 4,511,069. Such formulations may be conveniently prepared by dissolving compositions according to the present invention in water to produce an aqueous solution, and rendering said solution sterile. The formulations may be presented in multi-dose containers, for example in the sealed dispensing system disclosed in U.S. Pat. No. 4,511,069. Other suitable nasal spray delivery systems have been described in Transdermal Systemic Medication, Y. W. Chien Ed., Elsevier Publishers, New York, 1985; and in U.S. Pat. No. 4,778,810. Additional aerosol delivery forms may include, e.g., compressed air-, jet-, ultrasonic-, and piezoelectric nebulizers, which deliver the biologically active agent dissolved or suspended in a pharmaceutical solvent, e.g., water, ethanol, or a mixture thereof.

Nasal and pulmonary spray solutions of the present invention typically comprise the drug or drug to be delivered, optionally formulated with a surface active agent, such as a nonionic surfactant (e.g., polysorbate-80), and one or more buffers. In some embodiments of the present invention, the nasal spray solution further comprises a propellant. The pH of the nasal spray solution is optionally between about pH 6.8 and 7.2, but when desired the pH is adjusted to optimize delivery of a charged macromolecular species (e.g., a therapeutic protein or peptide) in a substantially unionized state. The pharmaceutical solvents employed can also be a slightly acidic aqueous buffer (pH 4-6). Suitable buffers for use within these compositions are as described above or as otherwise known in the art. Other components may be added to enhance or maintain chemical stability, including preservatives, surfactants, dispersants, or gases. Suitable preservatives include, but

are not limited to, phenol, methyl paraben, paraben, m-cresol, thiomersal, benzylalkonimum chloride, and the like. Suitable surfactants include, but are not limited to, oleic acid, sorbitan trioleate, polysorbates, lecithin, phosphotidyl cholines, and various long chain diglycerides and phospholipids. Suitable dispersants include, but are not limited to, ethylenediaminetetraacetic acid, and the like. Suitable gases include, but are not limited to, nitrogen, helium, chlorofluorocarbons (CFCs), hydrofluorocarbons (HFCs), carbon dioxide, air, and the like.

Within alternate embodiments, mucosal formulations are administered as dry powder formulations comprising the biologically active agent in a dry, usually lyophilized, form of an appropriate particle size, or within an appropriate particle size range, for intranasal delivery. Minimum particle size appropriate for deposition within the nasal or pulmonary passages is often about 0.5 μ mass median equivalent aerodynamic diameter (MMEAD), commonly about 1 μ MMEAD, and more typically about 2 μ MMEAD. Maximum particle size appropriate for deposition within the nasal passages is often about 10 μ MMEAD, commonly about 8 μ MMEAD, and more typically about 4 μ MMEAD. Intranasally respirable powders within these size ranges can be produced by a variety of conventional techniques, such as jet milling, spray drying, solvent precipitation, supercritical fluid condensation, and the like. These dry powders of appropriate MMEAD can be administered to a patient via a conventional dry powder inhaler (DPI) which rely on the patient's breath, upon pulmonary or nasal inhalation, to disperse the power into an aerosolized amount. Alternatively, the dry powder may be administered via air assisted devices that use an external power source to disperse the powder into an aerosolized amount, e.g., a piston pump.

Dry powder devices typically require a powder mass in the range from about 1 mg to 20 mg to produce a single aerosolized dose ("puff"). If the required or desired dose of the biologically active agent is lower than this amount, the powdered active agent will typically be combined with a pharmaceutical dry bulking powder to provide the required total powder mass. Preferred dry bulking powders include sucrose, lactose, dextrose, mannitol, glycine, trehalose, human serum albumin (HSA), and starch. Other suitable dry bulking powders include cellobiose, dextrans, maltotriose, pectin, sodium citrate, sodium ascorbate, and the like.

To formulate compositions for mucosal delivery within the present invention, the biologically active agent can be combined with various pharmaceutically acceptable additives, as well as a base or carrier for dispersion of the active agent(s). Desired additives include, but are not limited to, pH control agents, such as arginine, sodium hydroxide, glycine, hydrochloric acid, citric acid, etc. In addition, local anesthetics (e.g., benzyl alcohol), isotonizing agents (e.g., sodium chloride, mannitol, sorbitol), adsorption inhibitors (e.g., Tween 80), solubility enhancing agents (e.g., cyclodextrins and derivatives thereof), stabilizers (e.g., serum albumin), and reducing agents (e.g., glutathione) can be included. When the composition for mucosal delivery is a liquid, the tonicity of the formulation, as measured with reference to the tonicity of 0.9% (w/v) physiological saline solution taken as unity, is typically adjusted to a value at which no substantial, irreversible tissue damage will be induced in the nasal mucosa at the site of administration. Generally, the tonicity of the solution is adjusted to a value of about 1/3 to 3, more typically 1/2 to 2, and most often 3/4 to 1.7.

The biologically active agent may be dispersed in a base or vehicle, which may comprise a hydrophilic compound having a capacity to disperse the active agent and any desired additives. The base may be selected from a wide range of suitable carriers, including but not limited to, copolymers of polycarboxylic acids or salts thereof, carboxylic anhydrides (e.g. maleic anhydride) with other monomers (e.g. methyl (meth)acrylate, acrylic acid, etc.), hydrophilic vinyl polymers such as polyvinyl acetate, polyvinyl alcohol, polyvinylpyrrolidone, cellulose derivatives such as hydroxymethylcellulose, hydroxypropylcellulose, etc., and natural polymers such as chitosan, collagen, sodium alginate, gelatin, hyaluronic acid, and nontoxic metal salts thereof. Often, a biodegradable polymer is selected as a base or carrier, for example, polylactic acid, poly(lactic acid-glycolic acid) copolymer, polyhydroxybutyric acid, poly(hydroxybutyric acid-glycolic acid) copolymer and mixtures thereof. Alternatively or additionally, synthetic fatty acid esters such as polyglycerin fatty acid esters, sucrose fatty acid esters, etc. can be employed as carriers. Hydrophilic polymers and other carriers can be used alone or in combination, and enhanced structural integrity can be imparted to the carrier by partial crystallization, ionic bonding, crosslinking and the like. The carrier can be provided in a variety of forms, including, fluid or viscous solutions, gels, pastes, powders, microspheres and films for direct application to the nasal mucosa. The use

of a selected carrier in this context may result in promotion of absorption of the biologically active agent.

The biologically active agent can be combined with the base or carrier according to a variety of methods, and release of the active agent may be by diffusion, disintegration of the carrier, or associated formulation of water channels. In some circumstances, the active agent is dispersed in microcapsules (microspheres) or nanocapsules (nanospheres) prepared from a suitable polymer, e.g., isobutyl 2-cyanoacrylate (see, e.g., Michael et al., J. Pharmacy Pharmacol. 43: 1-5, 1991), and dispersed in a biocompatible dispersing medium applied to the nasal mucosa, which yields sustained delivery and biological activity over a protracted time.

To further enhance mucosal delivery of pharmaceutical agents within the invention, formulations comprising the active agent may also contain a hydrophilic low molecular weight compound as a base or excipient. Such hydrophilic low molecular weight compounds provide a passage medium through which a water-soluble active agent, such as a physiologically active peptide or protein, may diffuse through the base to the body surface where the active agent is absorbed. The hydrophilic low molecular weight compound optionally absorbs moisture from the mucosa or the administration atmosphere and dissolves the water-soluble active peptide. The molecular weight of the hydrophilic low molecular weight compound is generally not more than 10000 and preferably not more than 3000. Exemplary hydrophilic low molecular weight compound include polyol compounds, such as oligo-, di- and monosaccarides such as sucrose, mannitol, lactose, L-arabinose, D-erythrose, D-ribose, D-xylose, D-mannose, D-galactose, lactulose, cellobiose, gentibiose, glycerin and polyethylene glycol. Other examples of hydrophilic low molecular weight compounds useful as carriers within the invention include N-methylpyrrolidone, and alcohols (e.g. oligovinyl alcohol, ethanol, ethylene glycol, propylene glycol, etc.) These hydrophilic low molecular weight compounds can be used alone or in combination with one another or with other active or inactive components of the intranasal formulation.

The compositions of the invention may alternatively contain as pharmaceutically acceptable carriers substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate,

triethanolamine oleate, etc. For solid compositions, conventional nontoxic pharmaceutically acceptable carriers can be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like.

Therapeutic compositions for administering the biologically active agent can also be formulated as a solution, microemulsion, or other ordered structure suitable for high concentration of active ingredients. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. Proper fluidity for solutions can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of a desired particle size in the case of dispersible formulations, and by the use of surfactants. In many cases, it will be desirable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, or sodium chloride in the composition. Prolonged absorption of the biologically active agent can be brought about by including in the composition an agent which delays absorption, for example, monostearate salts and gelatin.

In certain embodiments of the invention, the biologically active agent is administered in a time release formulation, for example in a composition which includes a slow release polymer. The active agent can be prepared with carriers that will protect against rapid release, for example a controlled release vehicle such as a polymer, microencapsulated delivery system or bioadhesive gel. Prolonged delivery of the active agent, in various compositions of the invention can be brought about by including in the composition agents that delay absorption, for example, aluminum monosterate hydrogels and gelatin. When controlled release formulations of the biologically active agent is desired, controlled release binders suitable for use in accordance with the invention include any biocompatible controlled-release material which is inert to the active agent and which is capable of incorporating the biologically active agent. Numerous such materials are known in the art. Useful controlled-release binders are materials that are metabolized slowly under physiological conditions following their intranasal delivery (e.g., at the nasal mucosal surface, or in the presence of bodily fluids following transmucosal delivery). Appropriate binders include but are not limited to biocompatible polymers and copolymers previously used in the art in sustained release formulations. Such biocompatible compounds are non-toxic and inert to surrounding tissues, and do not

trigger significant adverse side effects such as nasal irritation, immune response, inflammation, or the like. They are metabolized into metabolic products that are also biocompatible and easily eliminated from the body.

Exemplary polymeric materials for use in this context include, but are not limited to, polymeric matrices derived from copolymeric and homopolymeric polyesters having hydrolysable ester linkages. A number of these are known in the art to be biodegradable and to lead to degradation products having no or low toxicity. Exemplary polymers include polyglycolic acids (PGA) and polylactic acids (PLA), poly(DL-lactic acid-co-glycolic acid)(DL PLGA), poly(D-lactic acid-coglycolic acid)(D PLGA) and poly(L-lactic acid-co-glycolic acid)(L PLGA). Other useful biodegradable or bioerodable polymers include but are not limited to such polymers as poly(epsilon-caprolactone), poly(epsilon-aprolactone-CO-lactic acid), poly(epsilon.-aprolactone-CO-glycolic acid), poly(beta-hydroxy butyric acid), poly(alkyl-2-cyanoacrilate), hydrogels such as poly(hydroxyethyl methacrylate), polyamides, poly(amino acids) (i.e., L-leucine, glutamic acid, L-aspartic acid and the like), poly (ester urea), poly (2-hydroxyethyl DL-aspartamide), polyacetal polymers, polyorthoesters, polycarbonate, polymaleamides, polysaccharides and copolymers thereof. Many methods for preparing such formulations are generally known to those skilled in the art. Other useful formulations include controlled-release compositions such as are known in the art for the administration of leuprolide (trade name: Lupron.RTM.), e.g., microcapsules (U.S. Pat. Nos. 4,652,441 and 4,917,893), lactic acid-glycolic acid copolymers useful in making microcapsules and other formulations (U.S. Pat. Nos. 4,677,191 and 4,728,721), and sustained-release compositions for water-soluble peptides (U.S. Pat. No. 4,675,189).

The mucosal formulations of the invention typically must be sterile and stable under all conditions of manufacture, storage and use. Sterile solutions can be prepared by incorporating the active compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders, methods of preparation include vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. The prevention

of the action of microorganisms can be accomplished by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like.

In more detailed aspects of the invention, the biologically active agent is stabilized to extend its effective half-life following delivery to the subject, particularly for extending metabolic persistence in an active state within the physiological environment (e.g., at the nasal mucosal surface, in the bloodstream, or within a connective tissue compartment or fluid-filled body cavity). For this purpose, the biologically active agent may be modified by chemical means, e.g., chemical conjugation, N-terminal capping, PEGylation, or recombinant means, e.g., site-directed mutagenesis or construction of fusion proteins, or formulated with various stabilizing agents or carriers. Thus stabilized, the active agent administered as above retains biological activity for an extended period (e.g., 2-3, up to 5-10 fold greater stability) under physiological conditions compared to its non-stabilized form.

In accordance with the various treatment methods of the invention, the biologically active agent is delivered to a mammalian subject in a manner consistent with conventional methodologies associated with management of the disorder for which treatment or prevention is sought. In accordance with the disclosure herein, a prophylactically or therapeutically effective amount of the biologically active agent is administered to a subject in need of such treatment for a time and under conditions sufficient to prevent, inhibit, and/or ameliorate a selected disease or condition or one or more symptom(s) thereof.

The term "subject" as used herein means any mammalian patient to which the compositions of the invention may be administered. Typical subjects intended for treatment with the compositions and methods of the present invention include humans, as well as non-human primates and other animals. To identify subject patients for prophylaxis or treatment according to the methods of the invention, accepted screening methods are employed to determine risk factors associated with a targeted or suspected disease of condition as discussed above, or to determine the status of an existing disease or condition in a subject. These screening methods include, for example, conventional work-ups to determine familial, sexual, drug-use and other such risk factors that may be associated with the targeted or suspected disease or condition, as well as diagnostic methods such as various ELISA immunoassay methods, which are available and well known in the art to detect and/or

characterize disease-associated markers. These and other routine methods allow the clinician to select patients in need of therapy using the mucosal methods and formulations of the invention. In accordance with these methods and principles, biologically active agents may be mucosally administered according to the teachings herein as an independent prophylaxis or treatment program, or as a follow-up, adjunct or coordinate treatment regimen to other treatments, including surgery, vaccination, immunotherapy, hormone treatment, cell, tissue, or organ transplants, and the like.

Mucosal administration according to the invention allows effective self-administration of treatment by patients, provided that sufficient safeguards are in place to control and monitor dosing and side effects. Mucosal administration also overcomes certain drawbacks of other administration forms, such as injections, that are painful and expose the patient to possible infections and may present drug bioavailability problems. For nasal and pulmonary delivery, systems for controlled aerosol dispensing of therapeutic liquids as a spray are well known. In one embodiment, metered doses of active agent are delivered by means of a specially constructed mechanical pump valve (U.S. Pat. No. 4,511,069). This hand-held delivery device is uniquely nonvented so that sterility of the solution in the aerosol container is maintained indefinitely.

DOSAGE

For prophylactic and treatment purposes, the biologically active agent(s) disclosed herein may be administered to the subject in a single bolus delivery, via continuous delivery (e.g., continuous transdermal, mucosal, or intravenous delivery) over an extended time period, or in a repeated administration protocol (e.g., by an hourly, daily or weekly, repeated administration protocol). In this context, a therapeutically effective dosage of the biologically active agent(s) may include repeated doses within a prolonged prophylaxis or treatment regimen, that will yield clinically significant results to alleviate one or more symptoms or detectable conditions associated with a targeted disease or condition as set forth above. Determination of effective dosages in this context is typically based on animal model studies followed up by human clinical trials and is guided by determining effective dosages and administration protocols that significantly reduce the occurrence or

severity of targeted disease symptoms or conditions in the subject. Suitable models in this regard include, for example, murine, rat, porcine, feline, non-human primate, and other accepted animal model subjects known in the art. Alternatively, effective dosages can be determined using *in vitro* models (e.g., immunologic and histopathologic assays). Using such models, only ordinary calculations and adjustments are typically required to determine an appropriate concentration and dose to administer a therapeutically effective amount of the biologically active agent(s) (e.g., amounts that are intranasally effective, transdermally effective, intravenously effective, or intramuscularly effective to elicit a desired response). In alternative embodiments, an "effective amount" or "effective dose" of the biologically active agent(s) may simply inhibit or enhance one or more selected biological activity(ies) correlated with a disease or condition, as set forth above, for either therapeutic or diagnostic purposes.

The actual dosage of biologically active agents will of course vary according to factors such as the disease indication and particular status of the subject (e.g., the subject's age, size, fitness, extent of symptoms, susceptibility factors, etc), time and route of administration, other drugs or treatments being administered concurrently, as well as the specific pharmacology of the biologically active agent(s) for eliciting the desired activity or biological response in the subject. Dosage regimens may be adjusted to provide an optimum prophylactic or therapeutic response. A therapeutically effective amount is also one in which any toxic or detrimental side effects of the biologically active agent is outweighed in clinical terms by therapeutically beneficial effects. A non-limiting range for a therapeutically effective amount of a biologically active agent within the methods and formulations of the invention is 0.01 μg/kg-10 mg/kg, more typically between about 0.05 and 5 mg/kg, and in certain embodiments between about 0.2 and 2 mg/kg. Dosages within this range can be achieved by single or multiple administrations, including, e.g., multiple administrations per day, daily or weekly administrations. Per administration, it is desirable to administer at least one microgram of the biologically active agent (e.g., one or more JAM, occludin and claudin peptides, proteins, analogs and mimetics, and other biologically active agents), more typically between about 10 μg and 5.0 mg, and in certain embodiments between about 100 μg and 1.0 or 2.0 mg to an average human subject. It is to be further noted that for each particular subject, specific dosage

regimens should be evaluated and adjusted over time according to the individual need and professional judgment of the person administering or supervising the administration of the permeabilizing peptide(s) and other biologically active agent(s).

Dosage of biologically active agents may be varied by the attending clinician to maintain a desired concentration at the target site. For example, a selected local concentration of the biologically active agent in the bloodstream or CNS may be about 1-50 nanomoles per liter, sometimes between about 1.0 nanomole per liter and 10, 15 or 25 nanomoles per liter, depending on the subject's status and projected or measured response. Higher or lower concentrations may be selected based on the mode of delivery, e.g., trans-epidermal, rectal, oral, or intranasal delivery versus intravenous or subcutaneous delivery. Dosage should also be adjusted based on the release rate of the administered formulation, e.g., of a nasal spray versus powder, sustained release oral versus injected particulate or transdermal delivery formulations, etc. To achieve the same serum concentration level, for example, slow-release particles with a release rate of 5 nanomolar (under standard conditions) would be administered at about twice the dosage of particles with a release rate of 10 nanomolar.

Additional guidance as to particular dosages for selected biologically active agents for use within the invention may be found widely disseminated in the literature. This is true for many of the therapeutic peptide and protein agents disclosed herein. For example, guidance for administration of human growth hormone (hGH) in the treatment of individuals intoxicated with poisonous substances may be found in U.S. Pat. Nos. 5,140,008 and 4,816,439; guidance for administration of hGH in the treatment of topical ulcers may be found in U.S. Pat. No. 5,006,509; guidance for administration of GM-CSF, G-CSF, and multi-CSF for treatment of pancytopenia may be found in U.S. Pat. No. 5,198,417; guidance for delivery of asparaginase for treatment of neoplasms may be found in U.S. Pat. Nos. 4,478,822 and 4,474,752; guidance for administration of L-asparaginase in the treatment of tumors is found in U.S. Pat. No. 5,290,773; guidance for administration of prostaglandin E1, prostaglandin E2, prostaglandin F2 alpha, prostaglandin I2, pepsin, pancreatin, rennin, papain, trypsin, pancrelipase, chymopapain, bromelain, chymotrypsin, streptokinase, urokinase, tissue plasminogen activator, fibrinolysin, deoxyribonuclease, sutilains, collagenase, asparaginase, or heparin in topical formulations may be found in U.S. Pat. No. 5,260,066; guidance for the

administration of superoxide dismutase, glucocerebrosides, asparaginase, adenosine deaminase, interleukin (1,2,3,4,5,6,7), tissue necrosis factor (TNF-alpha or TNF-beta), and colony stimulating factors (CSF, G-CSF, GM-CSF) in liposomes may be found in U.S. Pat. No. 5,225,212; guidance for administration of asparaginase in the treatment of neoplastic lesions may be found in U.S. Pat. No. 4,978,332; guidance for administration of asparaginase in the reduction of tumor growth may be found in U.S. Pat. No. 4,863,910; guidance for the administration of antibodies in the prevention of transplant rejection may be found in U.S. Pat. Nos. 4,657,760 and 5,654,210; guidance for the administration of interleukin-1 as a therapy for immunomodulatory conditions including T cell mutagenesis, induction of cytotoxic T cells, augmentation of natural killer cell activity, induction of interferon-gamma, restoration or enhancement of cellular immunity, and augmentation of cell-mediated anti-tumor activity may be found in U.S. Pat. No. 5,206,344; guidance for the administration of interleukin-2 in the treatment of tumors may be found in U.S. Pat. No. 4,690,915; and guidance for administration of interleukin-3 in the stimulation of hematopoiesis, as a cancer chemotherapy, and in the treatment of immune disorders may be found in U.S. Pat. No. 5,166,322. Each of the foregoing U.S. patents is with respect to the guidance provided for formulation and administration of particular biologically active agents therein).

KITS

The instant invention also includes kits, packages and multicontainer units containing the above described pharmaceutical compositions, active ingredients, and/or means for administering the same for use in the prevention and treatment of diseases and other conditions in mammalian subjects. Briefly, these kits include a container or formulation that contains one or more JAM, occludin and claudin peptides, proteins, analogs and mimetics, and other biologically active agents disclosed herein formulated in a pharmaceutical preparation for mucosal delivery. The biologically active agent(s) is/are optionally contained in a bulk dispensing container or unit or multi-unit dosage form. Optional dispensing means may be provided, for example a pulmonary or intranasal spray applicator. Packaging materials optionally include a label or instruction indicating that the pharmaceutical

agent packaged therewith can be used mucosally, e.g., intranasally, for treating or preventing a specific disease or condition. In more detailed embodiments of the invention, kits include one or more mucosal delivery-enhancing agents selected from: (a) aggregation inhibitory agents; (b) charge modifying agents; (c) pH control agents; (d) degradative enzyme inhibitors; (e) mucolytic or mucus clearing agents; (f) ciliostatic agents; (g) membrane penetration-enhancing agents (e.g., (i) a surfactant, (ii) a bile salt, (ii) a phospholipid or fatty acid additive, mixed micelle, liposome, or carrier, (iii) an alcohol, (iv) an enamine, (v) an NO donor compound, (vi) a long-chain amphipathic molecule (vii) a small hydrophobic penetration enhancer; (viii) sodium or a salicylic acid derivative; (ix) a glycerol ester of acetoacetic acid (x) a cyclodextrin or beta-cyclodextrin derivative, (xi) a medium-chain fatty acid, (xii) a chelating agent, (xiii) an amino acid or salt thereof, (xiv) an N-acetylamino acid or salt thereof, (xv) an enzyme degradative to a selected membrane component, (ix) an inhibitor of fatty acid synthesis, (x) an inhibitor of cholesterol synthesis; or (xi) any combination of the membrane penetration enhancing agents of (i)-(x)); (h) secondary modulatory agents of epithelial junction physiology, such as nitric oxide (NO) stimulators, chitosan, and chitosan derivatives; (i) vasodilator agents; (j) selective transport-enhancing agents; and (k) stabilizing delivery vehicles, carriers, supports or complex-forming species with which the biologically active agent is/are effectively combined, associated, contained, encapsulated or bound to stabilize the active agent for enhanced mucosal delivery.

The invention is further illustrated by the following specific examples that are not intended in any way to limit the scope of the invention.

EXAMPLE I

Mucosal Delivery - Permeation Kinetics and Cytotoxicity

1. Organotypic Model

The following methods are generally useful for evaluating mucosal delivery parameters, kinetics and side effects for a biologically active therapeutic agent and a mucosal delivery-enhancing effective amount of a permeabilizing peptide that reversibly enhances mucosal epithelial paracellular transport by modulating epithelial junctional structure and/or physiology in a mammalian subject. The permeabilizing

peptide generally effectively inhibits homotypic binding of an epithelial membrane adhesive protein selected from a junctional adhesion molecule (JAM), occludin, or claudin protein. The permeabilizing peptide is from about 4 to 25 contiguous amino acids (or alternatively, from about 6-15 contiguous amino acids) of an extracellular domain of a mammalian junctional adhesion molecule (JAM), e.g., JAM-1, JAM-2, or JAM-3 , an extracellular domain of mammalian claudin, e.g., claudin-1, claudin-2, claudin-3, claudin-4, claudin-5, claudin-6, claudin-7, claudin-8, claudin-9, or claudin-10, or an extracellular domain of mammalian occludin, within the formulations and method of the invention.

Permeation kinetics and cytotoxicity are also useful for determining the efficacy and characteristics of the various mucosal delivery-enhancing agents disclosed herein for combinatorial formulation or coordinate administration with a permeabilizing peptide comprising an extracellular domain of a mammalian JAM, mammalian claudin, or mammalian occludin protein. In one exemplary protocol, permeation kinetics and lack of unacceptable cytotoxicity are demonstrated for an intranasal delivery-enhancing effective amount of permeabilizing peptides as disclosed above in combination with a biologically active therapeutic agent, exemplified by interferon-β.

The EpiAirway™ system was developed by MatTek Corp (Ashland, MA) as a model of the pseudostratified epithelium lining the respiratory tract. The epithelial cells are grown on porous membrane-bottomed cell culture inserts at an air-liquid interface, which results in differentiation of the cells to a highly polarized morphology. The apical surface is ciliated with a microvillous ultrastructure and the epithelium produces mucus (the presence of mucin has been confirmed by immunoblotting). The inserts have a diameter of 0.875 cm, providing a surface area of 0.6 cm^2. The cells are plated onto the inserts at the factory approximately three weeks before shipping. One "kit" consists of 24 units.

A. On arrival, the units are placed onto sterile supports in 6-well microplates. Each well receives 5 mL of proprietary culture medium. This DMEM-based medium is serum free but is supplemented with epidermal growth factor and other factors. The medium is always tested for endogenous levels of any cytokine or growth factor which is being considered for intranasal delivery, but has been free of all cytokines and factors studied to date except insulin. The 5 mL volume is just

sufficient to provide contact to the bottoms of the units on their stands, but the apical surface of the epithelium is allowed to remain in direct contact with air. Sterile tweezers are used in this step and in all subsequent steps involving transfer of units to liquid-containing wells to ensure that no air is trapped between the bottoms of the units and the medium.

B. The units in their plates are maintained at 37°C in an incubator in an atmosphere of 5% CO_2 in air for 24 hours. At the end of this time the medium is replaced with fresh medium and the units are returned to the incubator for another 24 hours.

2. Experimental Protocol - Permeation Kinetics

A. A "kit" of 24 EpiAirway™ units can routinely be employed for evaluating five different formulations, each of which is applied to quadruplicate wells. Each well is employed for determination of permeation kinetics (4 time points), transepithelial electrical resistance (TER), mitochondrial reductase activity as measured by MTT reduction, and cytolysis as measured by release of LDH. An additional set of wells is employed as controls, which are sham treated during determination of permeation kinetics, but are otherwise handled identically to the test sample-containing units for determinations of transepithelial resistance and viability. The determinations on the controls are routinely also made on quadruplicate units, but occasionally we have employed triplicate units for the controls and have dedicated the remaining four units in the kit to measurements of transepithelial resistance and viability on untreated units or we have frozen and thawed the units for determinations of total LDH levels to serve as a reference for 100% cytolysis.

B. In all experiments, the mucosal delivery formulation to be studied is applied to the apical surface of each unit in a volume of 100 μL, which is sufficient to cover the entire apical surface. An appropriate volume of the test formulation at the concentration applied to the apical surface (no more than 100 μL is generally needed) is set aside for subsequent determination of concentration of the active material by ELISA or other designated assay.

C. The units are placed in 6 well plates without stands for the experiment: each well contains 0.9 mL of medium which is sufficient to contact the porous membrane bottom of the unit but does not generate any significant upward hydrostatic pressure on the unit.

D. In order to minimize potential sources of error and avoid any formation of concentration gradients, the units are transferred from one 0.9 mL-containing well to another at each time point in the study. These transfers are made at the following time points, based on a zero time at which the 100 μL volume of test material was applied to the apical surface: 15 minutes, 30 minutes, 60 minutes, and 120 minutes.

E. In between time points the units in their plates are kept in the 37°C incubator. Plates containing 0.9 mL medium per well are also maintained in the incubator so that minimal change in temperature occurs during the brief periods when the plates are removed and the units are transferred from one well to another using sterile forceps.

F. At the completion of each time point, the medium is removed from the well from which each unit was transferred, and aliquotted into two tubes (one tube receives 700 μL and the other 200 μL) for determination of the concentration of permeated test material and, in the event that the test material is cytotoxic, for release of the cytosolic enzyme, lactate dehydrogenase, from the epithelium. These samples are kept in the refrigerator if the assays are to be conducted within 24 hours, or the samples are subaliquotted and kept frozen at -80°C until thawed once for assays. Repeated freeze-thaw cycles are to be avoided.

G. In order to minimize errors, all tubes, plates, and wells are prelabeled before initiating an experiment.

H. At the end of the 120 minute time point, the units are transferred from the last of the 0.9 mL containing wells to 24-well microplates, containing 0.3 mL medium per well. This volume is again sufficient to contact the bottoms of the units, but not to exert upward hydrostatic pressure on the units. The units are returned to the incubator prior to measurement of transepithelial resistance.

3. Experimental Protocol - Transepithelial Electrical Resistance

A. Respiratory airway epithelial cells form tight junctions *in vivo* as well as *in vitro*, and thereby restrict the flow of solutes across the tissue. These junctions confer a transepithelial resistance of several hundred ohms x cm^2 in excised airway tissues. In the MatTek EpiAirway™ units, the transepithelial electrical resistance (TER) is reported by the manufacturer to be routinely around 1000 ohms x cm^2. Data determined herein indicates that the TER of control EpiAirway™ units

which have been sham-exposed during the sequence of steps in the permeation study is somewhat lower (700-800 ohms x cm^2), but, since permeation of small molecules is proportional to the inverse of the TER, this value is still sufficiently high to provide a substantial barrier to permeation. The porous membrane-bottomed units without cells, conversely, provide only minimal transmembrane resistance (approximately 5-20 ohms x cm^2).

B. Accurate determinations of TER require that the electrodes of the ohmmeter be positioned over a significant surface area above and below the membrane, and that the distance of the electrodes from the membrane be reproducibly controlled. The method for TER determination recommended by MatTek and employed for all experiments herein employs an "EVOM"™ epithelial voltohmmeter and an "ENDOHM"™ tissue resistance measurement chamber from World Precision Instruments, Inc., Sarasota, FL.

C. The chamber is initially filled with Dulbecco's phosphate buffered saline (PBS) for at least 20 minutes prior to TER determinations in order to equilibrate the electrodes.

D. Determinations of TER are made with 1.5 mL of PBS in the chamber and 350 μL of PBS in the membrane-bottomed unit being measured. The top electrode is adjusted to a position just above the membrane of a unit containing no cells (but containing 350 μL of PBS) and then fixed to ensure reproducible positioning. The resistance of a cell-free unit is typically 5-20 ohms x cm^2 ("background resistance").

E. Once the chamber is prepared and the background resistance is recorded, units in a 24-well plate that had just been employed in permeation determinations are removed from the incubator and individually placed in the chamber for TER determinations.

F. Each unit is first transferred to a petri dish containing PBS to ensure that the membrane bottom is moistened. An aliquot of 350 μL PBS is added to the unit and then carefully aspirated into a labeled tube to rinse the apical surface. A second wash of 350 μL PBS is then applied to the unit and aspirated into the same collection tube.

G. The unit is gently blotted free of excess PBS on its exterior surface only before being placed into the chamber (containing a fresh 1.5 mL aliquot of PBS).

An aliquot of 350 μL PBS is added to the unit before the top electrode is placed on the chamber and the TER is read on the EVOM meter.

H. After the TER of the unit is read in the ENDOHM chamber, the unit is removed, the PBS is aspirated and saved, and the unit is returned with an air interface on the apical surface to a 24-well plate containing 0.3 mL medium per well.

I. The units are read in the following sequence: all sham-treated controls, followed by all formulation-treated samples, followed by a second TER reading of each of the sham-treated controls. After all the TER determinations are complete, the units in the 24-well microplate are returned to the incubator for determination of viability by MTT reduction.

4. Experimental Protocol - Viability by MTT Reduction

MTT is a cell-permeable tetrazolium salt which is reduced by mitochondrial dehydrogenase activity to an insoluble colored formazan by viable cells with intact mitochondrial function or by nonmitochondrial NAD(P)H dehydrogenase activity from cells capable of generating a respiratory burst. Formation of formazan is a good indicator of viability of epithelial cells since these cells do not generate a significant respiratory burst. We have employed a MTT reagent kit prepared by MatTek Corp for their units in order to assess viability.

A. The MTT reagent is supplied as a concentrate and is diluted into a proprietary DMEM-based diluent on the day viability is to be assayed (typically the afternoon of the day in which permeation kinetics and TER were determined in the morning). The final MTT concentration is 1 mg/mL

B. The final MTT solution is added to wells of a 24-well microplate at a volume of 300 μL per well. As has been noted above, this volume is sufficient to contact the membranes of the EpiAirway™ units but imposes no significant positive hydrostatic pressure on the cells.

C. The units are removed from the 24-well plate in which they were placed after TER measurements, and after removing any excess liquid from the exterior surface of the units, they are transferred to the plate containing MTT reagent. The units in the plate are then placed in an incubator at 37°C in an atmosphere of 5% CO_2 in air for 3 hours.

D. At the end of the 3-hour incubation, the units containing viable cells will have turned visibly purple. The insoluble formazan must be extracted from

the cells in their units to quantitate the extent of MTT reduction. Extraction of the formazan is accomplished by transferring the units to a 24-well microplate containing 2 mL extractant solution per well, after removing excess liquid from the exterior surface of the units as before. This volume is sufficient to completely cover both the membrane and the apical surface of the units. Extraction is allowed to proceed overnight at room temperature in a light-tight chamber. MTT extractants traditionally contain high concentrations of detergent, and destroy the cells.

E. At the end of the extraction, the fluid from within each unit and the fluid in its surrounding well are combined and transferred to a tube for subsequent aliquotting into a 96-well microplate (200 μL aliquots are optimal) and determination of absorbance at 570 nm on a VMax multiwell microplate spectrophotometer. To ensure that turbidity from debris coming from the extracted units does not contribute to the absorbance, the absorbance at 650 nm is also determined for each well in the VMax and is automatically subtracted from the absorbance at 570 nm. The "blank" for the determination of formazan absorbance is a 200 μL aliquot of extractant to which no unit had been exposed. This absorbance value is assumed to constitute zero viability.

F. Two units from each kit of 24 EpiAirway™ units are left untreated during determination of permeation kinetics and TER. These units are employed as the positive control for 100% cell viability. In all the studies conducted, there was no statistically significant difference in the viability of the cells in these untreated cells compared to viability of cells in control units which had been sham treated for permeation kinetics and on which TER determinations had been performed. The absorbance of all units treated with test formulations is assumed to be linearly proportional to the percent viability of the cells in the units at the time of the incubation with MTT. It should be noted that this assay is carried out typically no sooner than four hours after introduction of the test material to the apical surface, and subsequent to rinsing of the apical surface of the units during TER determination.

5. Determination of Viability by LDH Release

While measurement of mitochondrial reductase activity by MTT reduction is a sensitive probe of cell viability, the assay necessarily destroys the cells and therefore can be carried out only at the end of each study. When cells undergo necrotic lysis, their cytotosolic contents are spilled into the surrounding medium, and cytosolic

enzymes such as lactate dehydrogenase (LDH) can be detected in this medium. An assay for LDH in the medium can be performed on samples of medium removed at each time point of the two-hour determination of permeation kinetics. Thus, cytotoxic effects of formulations that do not develop until significant time has passed can be detected as well as effects of formulations that induce cytolysis with the first few minutes of exposure to airway epithelium.

A. The recommended LDH assay for evaluating cytolysis of the EpiAirway™ units is based on conversion of lactate to pyruvate with generation of NADH from NAD. The NADH is then reoxidized along with simultaneous reduction of the tetrazolium salt INT, catalyzed by a crude "diaphorase" preparation. The formazan formed from reduction of INT is soluble, so that the entire assay for LDH activity can be carried out in a homogenous aqueous medium containing lactate, NAD, diaphorase, and INT.

B. The assay for LDH activity is carried out on 50 μL aliquots from samples of "supernatant" medium surrounding an EpiAirway™ unit and collected at each time point. These samples were either stored for no longer than 24 h in the refrigerator or were thawed after being frozen within a few hours after collection. Each EpiAirway™ unit generates samples of supernatant medium collected at 15 min, 30 min, 1 h, and 2 h after application of the test material. The aliquots are all transferred to a 96 well microplate.

C. A 50 μL aliquot of medium that had not been exposed to a unit serves as a "blank" or negative control of 0% cytotoxicity. The apparent level of "endogenous" LDH present after reaction of the assay reagent mixture with the unexposed medium is the same within experimental error as the apparent level of LDH released by all the sham-treated control units over the entire time course of 2 hours required to conduct a permeation kinetics study. Thus, within experimental error, these sham-treated units show no cytolysis of the epithelial cells over the time course of the permeation kinetics measurements.

D. To prepare a sample of supernatant medium reflecting the level of LDH released after 100% of the cells in a unit have lysed, a unit which had not been subjected to any prior manipulations is added to a well of a 6-well microplate containing 0.9 mL of medium as in the protocol for determination of permeation kinetics, the plate containing the unit is frozen at -80°C, and the contents of the well

are then allowed to thaw. This freeze-thaw cycle effectively lyses the cells and releases their cytosolic contents, including LDH, into the supernatant medium. A 50 μL aliquot of the medium from the frozen and thawed cells is added to the 96-well plate as a positive control reflecting 100% cytotoxicity.

E. To each well containing an aliquot of supernatant medium, a 50 μL aliquot of the LDH assay reagent is added. The plate is then incubated for 30 minutes in the dark.

F. The reactions are terminated by addition of a "stop" solution of 1 M acetic acid, and within one hour of addition of the stop solution, the absorbance of the plate is determined at 490 nm.

G. Computation of percent cytolysis is based on the assumption of a linear relationship between absorbance and cytolysis, with the absorbance obtained from the medium alone serving as a reference for 0% cytolysis and the absorbance obtained from the medium surrounding a frozen and thawed unit serving as a reference for 100% cytolysis.

6. ELISA Determinations

The procedures for determining the concentrations of biologically active agents as test materials for evaluating enhanced permeation of active agents in conjunction with coordinate administration of a permeabilizing peptide or combinatorial formulation of the invention are generally as described above and in accordance with known methods and specific manufacturer instructions of ELISA kits employed for each particular assay. Permeation kinetics of the biologically active agent is generally determined by taking measurements at multiple time points (for example 15 min., 30 min., 60 min. and 120 min) after the biologically active agent is contacted with the apical epithelial cell surface (which may be simultaneous with, or subsequent to, exposure of the apical cell surface to the permeabilizing peptide(s)).

EpiAirway™ tissue membranes are cultured in phenol red and hydrocortisone free medium (MatTek Corp., Ashland, MA). The tissue membranes are cultured at 37°C for 48 hours to allow the tissues to equilibrate. Each tissue membrane is placed in an individual well of a 6-well plate containing 0.9 mL of serum free medium. 100 μL of the formulation (test sample or control) is applied to the apical surface of the membrane. Triplicate or quadruplicate samples of each test sample (permeabilizing peptide (typically at concentration of 1.0 mM permeabilizing peptide of JAM, claudin

or occludin; generally, within a concentration range from approximately 0.1 mM to approximately 1.2 mM permeabilizing peptide in combination with a biologically active agent, (e.g., interferon-β) and control (biologically active agent, interferon-β, alone) are evaluated in each assay. At each time point (15, 30, 60 and 120 minutes) the tissue membranes are moved to new wells containing fresh medium. The underlying 0.9 mL medium samples is harvested at each time point and stored at 4°C for use in ELISA and lactate dehydrogenase (LDH) assays.

The ELISA kits are typically two-step sandwich ELISAs: the immunoreactive form of the agent being studied is first "captured" by an antibody immobilized on a 96-well microplate and after washing unbound material out of the wells, a "detection" antibody is allowed to react with the bound immunoreactive agent. This detection antibody is typically conjugated to an enzyme (most often horseradish peroxidase) and the amount of enzyme bound to the plate in immune complexes is then measured by assaying its activity with a chromogenic reagent. In addition to samples of supernatant medium collected at each of the time points in the permeation kinetics studies, appropriately diluted samples of the formulation (i.e., containing the subject biologically active test agent) that was applied to the apical surface of the units at the start of the kinetics study are also assayed in the ELISA plate, along with a set of manufacturer-provided standards. Each supernatant medium sample is generally assayed in duplicate wells by ELISA (it will be recalled that quadruplicate units are employed for each formulation in a permeation kinetics determination, generating a total of sixteen samples of supernatant medium collected over all four time points).

A. It is not uncommon for the apparent concentrations of active test agent in samples of supernatant medium or in diluted samples of material applied to the apical surface of the units to lie outside the range of concentrations of the standards after completion of an ELISA. No concentrations of material present in experimental samples are determined by extrapolation beyond the concentrations of the standards; rather, samples are rediluted appropriately to generate concentrations of the test material which can be more accurately determined by interpolation between the standards in a repeat ELISA.

B. The ELISA for a biologically active test agent, for example, interferon-β, is unique in its design and recommended protocol. Unlike most kits, the ELISA employs two monoclonal antibodies, one for capture and another, directed

towards a nonoverlapping determinant for the biologically active test agent, e.g., interferon-β, as the detection antibody (this antibody is conjugated to horseradish peroxidase). As long as concentrations of the test agent that lie below the upper limit of the assay are present in experimental samples, the assay protocol can be employed as per the manufacturer's instructions, which allow for incubation of the samples on the ELISA plate with both antibodies present simultaneously. When the levels of test agent, e.g., interferon-β, in a sample are significantly higher than this upper limit, the levels of immunoreactive interferon-β may exceed the amounts of the antibodies in the incubation mixture, and some interferon-β which has no detection antibody bound will be captured on the plate, while some interferon-β which has detection antibody bound may not be captured. This leads to serious underestimation of interferon-β levels in the sample (it will appear that interferon-β levels in such a sample lie significantly below the upper limit of the assay). To eliminate this possibility, the assay protocol has been modified as follows:

B.1. The diluted samples are first incubated on the ELISA plate containing the immobilized capture antibody for one hour in the absence of any detection antibody. After the one hour incubation, the wells are washed free of unbound material.

B.2. The detection antibody is incubated with the plate for one hour to permit formation of immune complexes with all captured antigen. The concentration of detection antibody is sufficient to react with the maximum level of biologically active test agent which has been bound by the capture antibody. The plate is then washed again to remove any unbound detection antibody.

B.3. The peroxidase substrate is added to the plate and incubated for fifteen minutes to allow color development to take place.

B.4. The "stop" solution is added to the plate, and the absorbance is read at 450 nm as well as 490 nm in the VMax microplate spectrophotometer. The absorbance of the colored product at 490 nm is much lower than that at 450 nm, but the absorbance at each wavelength is still proportional to concentration of product. The two readings ensure that the absorbance is linearly related to the amount of bound biologically active test agent over the working range of the VMax instrument (we routinely restrict the range from 0 to 2.5 OD, although the instrument is reported to be accurate over a range from 0 to 3.0 OD). In the case

of interferon-β as the exemplary test agent, the levels of this compound in the samples is determined by interpolation between the OD values obtained for the different standards included in the ELISA. Samples with OD readings outside the range obtained for the standards are rediluted and run in a repeat ELISA.

RESULTS

Measurement of transepithelial resistance by TER Assay:

After the final assay time points, membranes were placed in individual wells of a 24 well culture plate in 0.3 mL of fresh medium and the transepithelial electrical resistance (TER) was measured using the EVOM Epithelial Voltohmmeter and an Endohm chamber (World Precision Instruments, Sarasota, FL). The top electrode was adjusted to be close to, but not in contact with, the top surface of the membrane. Tissues were removed, one at a time, from their respective wells and basal surfaces were rinsed by dipping in clean PBS. Apical surfaces were gently rinsed twice with PBS. The tissue unit was placed in the Endohm chamber, 250 μL of PBS added to the insert, the top electrode replaced and the resistance measured and recorded. Following measurement, the PBS was decanted and the tissue insert was returned to the culture plate. All TER values are reported as a function of the surface area of the tissue.

The final numbers were calculated as:

TER of cell membrane = (Resistance (R) of Insert with membrane – R of blank Insert) X Area of membrane (0.6 cm^2).

The effect of pharmaceutical formulations comprising intranasal delivery-enhancing agents, for example, permeabilizing peptides of JAM, claudin, or occludin, as measured by TER across the EpiAirway™ Cell Membrane (mucosal epithelial cell layer) is shown in Tables 10 and 11, below. Permeabilizing peptides of JAM, claudin, or occludin are applied to the EpiAirway™ Cell Membrane at a concentration of 1.0 mM. A decrease in TER value relative to the control value (control = approximately 1000 ohms-cm^2; normalized to 100.) indicates a decrease in cell membrane resistance and an increase in mucosal epithelial cell permeability.

Exemplary JAM-1 peptides NP-A, NP-B, NP-C, NP-D, NP-E, NP-8, and NP-21 (see Table 10) showed the greatest decrease in cell membrane resistance as

measured by TER among the candidate JAM peptides assayed. JAM-1 peptides NP-A, NP-B, NP-C, NP-D, NP-E, NP-8, and NP-21 exhibit decreased TER measurement as percentage of control to 47.5%, 58.4%, 78.2%, 74.8%, 79.6%, 82.5% and 76.6%, respectively (see Table 11). The results indicate that these exemplary peptides when contacted with a mucosal epithelium yield significant increases in mucosal epithelial cell permeability.

Exemplary Claudin peptides NP-10, NP-17, NP-27, NP-28, NP-29, NP-30, NP-31, NP-32, NP-33, NP-41, and NP-42 (see Table 10) from claudin-1, -2, -3, -4, and -5) showed the greatest decrease in cell membrane resistance as measured by TER among the candidate claudin peptides tested. Claudin peptides NP-10, NP-17, NP-27, NP-28, NP-29, NP-30, NP-31, NP-32, NP-33, NP-41, and NP-42 exhibit decreased TER measurement as percentage of control to 84.4%, 83.0%, 51.5%, 75.4%, 82.8%, 74.1%, 63.4%, 87.3%, 62.4%, 76.0%, and 76.9%, respectively (see Table 11). The results indicate that these exemplary peptides when contacted with a mucosal epithelium yield significant increases in mucosal epithelial cell permeability.

Exemplary occludin peptides NP-22, NP-23, NP-24, NP-25, and NP-26 showed the greatest decrease in cell membrane resistance as measured by TER among the candidate occludin peptides tested. The results indicate that these exemplary formulations provide significant increases in mucosal epithelial cell permeability. Occludin peptides NP-22, NP-23, NP-24, NP-25, and NP-26 exhibit decreased TER measurement as percentage of control to 65.0%, 65.2%, 67.0%, 66.5%, and 63.7%, respectively. The results indicate that these exemplary peptides when contacted with a mucosal epithelium yield significant increases in mucosal epithelial cell permeability.

Table 10: Candidate Permeabilizing peptides of JAM, Claudin, or Occludin in Pharmaceutical Formulations Comprising Intranasal Delivery-Enhancing Agents

		SEQ ID NO:	
NP-A	VRIP	4	JAM-1 extracellular domain
NP-B	VKLSCAY	5	JAM-1 extracellular domain
NP-C	TGITFKSVT	6	JAM-1 extracellular domain
NP-D	ITAS	7	JAM-1 extracellular domain
NP-E	SVTR	8	JAM-1 extracellular domain
NP-1	SVTVHSSEP	847	JAM-1 extracellular domain
NP-2	KVFDSLLNLS	848	Claudin-1: 1st extracellular domain
NP-3	DRGYGTSLL	849	Occludin: 1st extracellular domain
NP-4	GYGYGYGYG	850	Occludin: 1st extracellular domain
NP-5	GSGFGSYGS	851	Occludin: 1st extracellular domain
NP-6	KFDQGDTTR	852	JAM-1 extracellular domain
NP-7	KVYDSLLALP	853	Claudin-3: 1st extracellular domain
NP-8	EDTGTYTCM	9	JAM-1 extracellular domain
NP-9	GEVKVKLIV	854	JAM-1 extracellular domain
NP-10	NTIIRDFYNP	54	Claudin-3: 2nd extracellular domain
NP-11	NRIVQEFYDP	855	Claudin-1: 2nd extracellular domain
NP-12	VSEEGGNSY	856	JAM-1 extracellular domain
NP-13	LVCYNNKIT	857	JAM-1 extracellular domain
NP-14	IVVREFYDPS	858	Claudin-5: 2nd extracellular domain
NP-15	YGYGGYTDP	859	Occludin: 1st extracellular domain
NP-16	VVQSTGHMQC	860	Claudin-5: 1st extracellular domain
NP-17	YAGDNIVTAQ	861	Claudin-1: 1st extracellular domain
NP-18	VSQSTGQIQC	862	Claudin-1: 1st extracellular domain
NP-19	YVGASIVTAV	863	Claudin-2: 1st extracellular domain
NP-20	FLDHNIVTAQ	864	Claudin-5: 1st extracellular domain
NP-21	GFSSPRVEW	865	JAM-1 extracellular domain
NP-22	GVNPTAQSS	866	Occludin: 2nd extracellular domain
NP-23	GSLYGSQIY	867	Occludin: 2nd extracellular domain
NP-24	AATGLYVDQ	32	Occludin: 2nd extracellular domain
NP-25	ALCNQFYTP	35	Occludin: 2nd extracellular domain
NP-26	YLYHYCVVD	42	Occludin: 2nd extracellular domain
NP-27	GILRDFYSPL	53	Claudin-2: 2nd extracellular domain
NP-28	MTPVNARYEF	58	Claudin-1: 2nd extracellular domain
NP-29	VASGQKREMG	59	Claudin-4: 2nd extracellular domain
NP-30	VPDSMKFEIG	60	Claudin-2: 2nd extracellular domain
NP-31	NIIQDFYNPL	61	Claudin-4: 2nd extracellular domain
NP-32	VPVSQKYELG	869	Claudin-5: 2nd extracellular domain
NP-33	VVPEAQKREM	63	Claudin-3: 2nd extracellular domain
NP-34	NIWEGLWMNC	870	Claudin-3: 1st extracellular domain
NP-35	FIGSNIVTSQ	871	Claudin-4: 1st extracellular domain

NP-36	VVQSTGQMQC	872	Claudin-3: 1st extracellular domain
NP-37	FIGSNIITSQ	873	Claudin-3: 1st extracellular domain
NP-38	AMYEGLWMSC	874	Claudin-1 1st extracellular domain
NP-39	GGSVGYPYG	875	Occludin: 1st extracellular domain
NP-40	TIWEGLWMNC	876	Claudin-4: 1st extracellular domain
NP-41	DIYSTLLGLP	877	Claudin-2: 1st extracellular domain
NP-42	GFSLGLWMEC	878	Claudin-2: 1st extracellular domain
NP-43	KVYDSVLALS	879	Claudin-5: 1st extracellular domain
NP-44	ATHSTGITQC	880	Claudin-2: 1st extracellular domain
NP-45	TTWLGLWMSC	881	Claudin-1: 1st extracellular domain
	VLPPS	882	JAM-1 extracellular domain
	YEDRVTF	883	JAM-1 extracellular domain
	PRVEW	884	JAM-1 extracellular domain
	GFSKGLWMEC	885	Claudin-2: 1st extracellular domain
	TTWKGLWMSC	886	Claudin-5: 1st extracellular domain

Table 11: Effect of Permeabilizing Peptides of JAM, Claudin, and Occludin as Intranasal Delivery-Enhancing Agents in Pharmaceutical Formulations on Trans Epithelial Electrical Resistance (TER) in EpiAirway™ Cell Membrane

Permeabilizing Peptide Extracellular Domain		Average TER Value	% of Control
JAM-1	NP-A	339 ± 105	47.5
JAM-1	NP-B	417 ± 64	58.4
JAM-1	NP-C	558 ± 113	78.2
JAM-1	NP-D	534 ± 94	74.8
JAM-1	NP-E	568 ± 30	79.6
	CONTROL	714 ± 91	100.0
JAM-1	NP-1	606 ± 130	115.0
Claudin-1	NP-2	709 ± 28	134.5
Occludin	NP-3	766 ± 94	145.4
Occludin	NP-4	500 ± 42	94.9
Occludin	NP-5	520 ± 134	98.7
JAM-1	NP-6	793 ± 44	150.5
Claudin-3	NP-7	811 ± 87	153.9
	CONTROL	527 ± 119	100.0
JAM-1	NP-8	254 ± 20	82.5
Claudin-3	NP-10	260 ± 66	84.4
Claudin-1	NP-11	292 ± 20	94.8
JAM-1	NP-12	279 ± 27	90.6
JAM-1	NP-13	288 ± 51	93.5
Claudin-5	NP-14	286 ± 56	92.9
	CONTROL	308 ± 30	100.0
Occludin	NP-15	383 ± 79	103.5

Permeabilizing Peptide Extracellular Domain		Average TER Value	% of Control
Claudin-5	NP-16	386 ± 44	104.3
Claudin-1	NP-17	307 ± 34	83.0
Claudin-1	NP-18	439 ± 145	118.6
Claudin-2	NP-19	374 ± 57	101.1
Claudin-5	NP-20	341 ± 31	92.2
JAM-1	NP-9	342 ± 185	92.4
	CONTROL	370 ± 22	100.0
Occludin	NP-22	297 ± 40	65.0
Occludin	NP-23	298 ± 56	65.2
Occludin	NP-24	306 ± 58	67.0
Occludin	NP-25	304 ± 41	66.5
Occludin	NP-26	291 ± 89	63.7
	CONTROL	457 ± 146	100.0
Claudin-2	NP-27	260 ± 13	51.5
Claudin-1	NP-28	381 ± 122	75.4
Claudin-4	NP-29	418 ± 112	82.8
Claudin-2	NP-30	374 ± 52	74.1
Claudin-4	NP-31	320 ± 60	63.4
Claudin-5	NP-32	441 ± 32	87.3
Claudin-3	NP-33	315 ± 50	62.4
JAM-1	NP-21	387 ± 20	76.6
	CONTROL	505 ± 157	100.0
Claudin-3	NP-34	297 ± 40	65.0
Claudin-4	NP-35	298 ± 56	65.2
Claudin-3	NP-36	306 ± 58	67.0
Claudin-3	NP-37	304 ± 41	66.5
Claudin-1	NP-38	291 ± 89	63.7
Claudin-4	NP-40	457 ± 146	100.0
	CONTROL		
Claudin	NP-41	263 ± 142	76.0
Claudin	NP-42	266 ± 92	76.9
Claudin	NP-43	446 ± 85	128.9
Claudin	NP-44	464 ± 61	134.1
Claudin	NP-45	456 ± 162	131.8
Occludin	NP-39	442 ± 185	127.7
	CONTROL	346 ± 188	100.0

Permeation kinetics as measured by ELISA Assay:

The effect of pharmaceutical formulations comprising interferon-β-1a and intranasal delivery-enhancing agents, for example, formulations including permeabilizing peptides of JAM, claudin or occludin, on the permeation of interferon-β across the EpiAirway™ Cell Membrane (mucosal epithelial cell layer) is measured as described above. Permeation of interferon-β-1a across the EpiAirway™ Cell

Membrane is measured by ELISA assay. Permeabilizing peptides of JAM, claudin or occludin are generally present in the ELISA assay within a concentration range from approximately 0.1 mM to approximately 1.2 mM, or generally within a concentration range from approximately 0.5 mM to approximately 1.1 mM. Permeabilizing peptides of JAM, claudin or occludin are typically present in the ELISA assay at a concentration of 1.0 mM.

For the exemplary intranasal formulations of the present invention, JAM-1 peptides NP-A, NP-B, NP-C, NP-D, NP-E, NP-8, and NP-21 at a concentration of 1.0 mM, combined in a pharmaceutical formulation or coordinately administered with interferon-β (60 MIU; 300 μg) or another biologically active agent, will yield a significant increase in permeation of the biologically active agent. In typical embodiments, the increase in permeation will be two-fold, often five-fold, at times ten-fold, and up to 25-fold, 50-fold, or 100-fold or greater, compared to control values, e.g., as measured by ELISA assay measuring permeation across an EpiAirway™ Cell Membrane.

For additional exemplary intranasal formulations of the present invention, claudin peptides NP-10, NP-17, NP-27, NP-28, NP-29, NP-30, NP-31, NP-32, NP-33, NP-41, and NP-42 at a concentration of 1.0 mM, combined in a pharmaceutical formulation or coordinately administered with interferon-β (60 MIU; 300 μg) or another biologically active agent, will yield a significant increase in mucosal permeation of the biologically active agent. In typical embodiments, the increase in permeation will be two-fold, often five-fold, at times ten-fold, and up to 25-fold, 50-fold, or 100-fold or greater, compared to control values, e.g., as measured by ELISA assay measuring permeation across an EpiAirway™ Cell Membrane.

For other exemplary intranasal formulations of the present invention, occludin peptides NP-22, NP-23, NP-24, NP-25, and NP-26 at a concentration of 1.0 mM, combined in a pharmaceutical formulation or coordinately administered with interferon-β (60 MIU; 300 μg) or another biologically active agent, will yield a significant increase in permeation of the biologically active agent. In typical embodiments, the increase in permeation will be two-fold, often five-fold, at times ten-fold, and up to 25-fold, 50-fold, or 100-fold or greater, compared to control values, e.g., as measured by ELISA assay measuring permeation across an EpiAirway™ Cell Membrane.

For other exemplary intranasal formulations of the present invention, JAM, claudin, or occludin peptides, administered in combination with a pharmaceutical formulation of interferon-β or another biologically active agent will yield a significant increase in permeation when the JAM, claudin, or occludin peptide is administered 10 minutes, 20 minutes, or 30 minutes prior to administration of interferon-β or another biologically active agent compared to simultaneous administration of permeabilizing peptide and biologically active agent. In typical embodiments, the increase in permeation will be two-fold, often five-fold, at times ten-fold, and up to 25-fold, 50-fold, or 100-fold or greater, compared to control values, e.g., as measured by ELISA assay measuring permeation across an EpiAirway™ Cell Membrane.

MTT Assay:

The MTT assays were performed using MTT-100, MatTek kits. 300 mL of the MTT solution was added into each well. Tissue inserts were gently rinsed with clean PBS and placed in the MTT solution. The samples were incubated at 37°C for 3 hours. After incubation the cell culture inserts were then immersed with 2.0 mL of the extractant solution per well to completely cover each insert. The extraction plate was covered and sealed to reduce evaporation. Extraction proceeds overnight at RT in the dark. After the extraction period was complete, the extractant solution was mixed and pipetted into a 96-well microtiter plate. Triplicates of each sample were loaded, as well as extractant blanks. The optical density of the samples was then measured at 550 nm on an optical density plate reader (Molecular Devices).

The MTT assay on exemplary formulations of the present invention for enhanced mucosal delivery of a therapeutic biological agent, for example, interferon-β, are shown. The results for pharmaceutical formulations comprising permeabilizing peptides, for example, JAM-1 peptides NP-A, NP-B, NP-C, NP-D, NP-E, NP-8, and NP-21 (see Table 10), indicate that there is minimal toxic effect of these exemplary peptides on viability of the mucosal epithelial tissue. These exemplary formulations were not toxic as measured by MTT assay results at greater than 80% of control.

The results for pharmaceutical formulations comprising permeabilizing peptides, for example, claudin peptides NP-10, NP-17, NP-27, NP-28, NP-29, NP-30, NP-31, NP-32, NP-33, NP-41, and NP-42 (see Table 10), indicate that there is minimal toxic effect of these exemplary peptides on viability of the mucosal epithelial

tissue. These exemplary formulations were not toxic as measured by MTT assay results at greater than 80% of control.

The results for pharmaceutical formulations comprising permeabilizing peptides, for example, occludin peptides NP-22, NP-23, NP-24, NP-25, and NP-26, indicate that there is minimal toxic effect of these exemplary peptides on viability of the mucosal epithelial tissue. These exemplary formulations were not toxic as measured by MTT assay results at greater than 80% of control.

LDH Assay:

The LDH assays for exemplary permeabilizing peptide formulations of the invention for enhanced mucosal delivery of interferon-β-1a and other biologically active agents demonstrated that there are no significant toxic effects of exemplary embodiments, for example, pharmaceutical formulations comprising permeabilizing JAM-1 peptides NP-A, NP-B, NP-C, NP-D, NP-E, NP-8, and NP-21 (see Table 10). Similar results were determined for exemplary, permeabilizing claudin peptides NP-10, NP-17, NP-27, NP-28, NP-29, NP-30, NP-31, NP-32, NP-33, NP-41, and NP-42 (Table 10), which exhibited no significant adverse effects on viability of mucosal epithelial tissue. Likewise, LDH assays further indicated that there are no significant toxic effects of exemplary occludin peptides NP-22, NP-23, NP-24, NP-25, and NP-26 on viability of mucosal epithelial tissue.

EXAMPLE 2

Bioavailability and bioactivity of dosages of nasal formulations of interferon-β (IFN-β) with permeabilizing peptides administered to healthy male volunteers

The present example provides a non-blinded study to determine the uptake of intranasally administered interferon-β in combination with a mucosal delivery-enhancing effective amount of a permeabilizing peptide into the blood serum in healthy male volunteers. The permeabilizing peptide reversibly enhances mucosal epithelial paracellular transport by modulating epithelial junctional structure and/or physiology in a mammalian subject. The permeabilizing peptide generally effectively inhibits homotypic binding of an epithelial membrane adhesive protein selected from a junctional adhesion molecule (JAM), occludin, or claudin protein.

Table 12: Formulations comprising interferon-β-1a and intranasal delivery-enhancing agents.

Formulation	Composition	Quantity	
F9	Interferon-β-1a (300 μg)	60	MIU
	albumin human	15	mg
	dibasic sodium phosphate	5.7	mg
	monobasic sodium phosphate	1.2	mg
	sodium chloride	5.8	mg
	benzalkonium chloride	1.0	mg
	L-α-phosphatidylcholine didecanoyl	0.5	mg
	methyl- β -cyclodextrin	30.0	mg
	EDTA disodium	1.0	mg
	gelatin	5.0	mg
	purified water USP q.s. to	1.0	ml

Table 13: Formulations comprising interferon-β-1a and intranasal delivery-enhancing agents.

Formulation	Composition	Quantity	
F9	F9 (Formulation with Interferon-β-1a; 300 μg)	60	MIU
JAM 1	F9 (Interferon-β-1a Formulation)	60	MIU
	NP-A peptide	1.0	mM
CLAUDIN-2	F9 (Interferon-β-1a Formulation)	60	MIU
	NP-27 peptide	1.0	mM
OCCLUDIN	F9 (Interferon-β-1a Formulation)	60	MIU
	NP-26 peptide	1.0	mM

STUDY SYNOPSES.

The study involves administration of an intranasal effective amount of an exemplary formulation of the invention, Formulation F9 in the presence of a permeabilizing peptide, for example, JAM-1 NP-A peptide, occludin NP-26 peptide, and/or claudin-2 NP-27 peptide, as described above, to evaluate the absorption and tolerance of the interferon-β intranasal formulation by the subjects. See Tables 12

and 13. Formulations comprising permeabilizing peptides of JAM, occludin or claudin have a concentration range of permeabilizing peptide between approximately 0.1 mM and approximately 1.0 mM of JAM, occludin or claudin peptide in the formulation. The study is a single dose, parallel group pharmacokinetic/pharmacodynamic study to evaluate absorption and tolerance of interferon-β-1a by two routes of administration: intramuscular and intranasal. The objective of the study is to evaluate the absorption, tolerance and pharmacodynamic parameters of equimolar doses of an exemplary formulation of interferon-β-1a in combination with one or more intranasal delivery-enhancing agents of the present invention, for example, permeabilizing peptides of JAM, occludin or claudin, administered intranasally, versus interferon-β-1a (Avonex®, Biogen, Inc., or Rebif®, Serono) administered intramuscularly, subcutaneously, or in the presence or absence of one or more intranasal delivery-enhancing agents.

Protocol: 36 healthy male subjects, age 18-50, are generally enrolled in the study. Groups of six subjects typically receive either Formulation F9 (60 μg; 6.0 MIU; interferon-β-1a), Formulation JAM-1 NP-A (= F9 + 1.0 mM JAM-1 NP-A peptide); Formulation CLAUDIN-2 NP-27 (= F9 + 1.0 mM CLAUDIN-2 NP-27 peptide); or Formulation OCCLUDIN NP-26 (= F9 + 1.0 mM OCCLUDIN NP-26 peptide) delivered intranasally as two 0.1 mL sprays, each containing 30 μg/0.1 mL. Six subjects receive a single dose of 60 μg interferon-β-1a(Avonex®) delivered intramuscularly. Six subjects receive a single dose of 60 μg interferon-β-1a (Rebif®; Ares-Serono) delivered subcutaneously.

The study is conducted in compliance with Good Clinical Practice regulations and all necessary regulatory and Insitiutional Review Board approvals were in place prior to start of the study.

In accordance with the teachings herein, this and similar studies will be readily practiced to demonstrate the novel and surprising characteristics of the mucosal delivery formulations and methods of the invention. In particular, these studies will evince that formulations and methods of the invention involving the use of permeabilizing peptides of JAM, occludin or claudin offer many advantages in terms of improving delivery of macromolecular drugs into and across mucosal surfaces. In the exemplary case of interferon-β-1a, the importance of these methods and formulations is underscored by the fact that no non-injectable products of interferon-

β-1a are currently available. Pulmonary administration has achieved some success but has disadvantages including patient inconvenience and questionable pulmonary safety.

In accordance with the foregoing teachings Table 14, below, provides projected exemplary pharmacokinetic data for intranasal delivery of interferon-β-1a in a pharmaceutical formulation of the invention (e.g., Formulation F-9 plus permeabilizing peptides of JAM-1, claudin-2, or occludin) compared to Formulation F-9 without permeabilizing peptide, intramuscular, or subcutaneous delivery of interferon-β-1a (Avonex® or Rebif®). Maximum concentration of interferon-β in the blood serum (C_{max}) at 3 hours post dosing is projected to be approximately 6.0 IU/mL for intranasal delivery of JAM-1 NP-A Formulation; 5.6 IU/mL for intranasal delivery of Claudin-2 NP-27 Formulation, 4.5 IU/mL for intranasal delivery of Occludin NP-26 Formulation--compared to 5.1 IU/mL for subcutaneous delivery of interferon-β-1a (at 12 MIU dose) or 4.9 to 5.2 IU/mL for intramuscular delivery of interferon-β-1a (at 12 MIU dose).

Time to maximum serum concentration of interferon-β in the blood serum (t_{max}) is projected to be at least 5- to 10-fold faster for intranasal delivery of the formulation of the present invention compared to subcutaneous or intramuscular delivery of interferon-β-1a (Avonex® or Rebif®). In exemplary embodiments t_{max} for intranasal delivery of JAM-1 NP-A Formulation is projected to be approximately 0.3 hours, or 0.3 hours for intranasal delivery of Claudin-2 NP-27 Formulation, or 0.4 hours for intranasal delivery of Occludin NP-26 Formulation--compared to a t_{max} of 3 to 4 hours for intramuscular or subcutaneous administration of Avonex® or Rebif®.

The results in Table 14 exemplify bioavailability of interferon-β as measured by interferon-β pharmacodynamic markers, for example, β-2 microglobulin and neopterin achieved by the methods and formulations herein, e.g., as measured by area under the concentration curve (AUC) in blood serum, CNS, CSF or in another selected physiological compartment or target tissue. Bioavailability of interferon-β as measured by interferon-β markers will be, for example, approximately $AUC_{0\text{-}96\ hr}$ for β-2 microglobulin of approximately 200 μIU•hr/mL of blood plasma or CSF. Bioavailability of interferon-β as measured by interferon-β markers will be, for example, approximately $AUC_{0\text{-}96\ hr}$ for neopterin of approximately 200 ng·hr/ml of blood plasma or CSF.

In accordance with the teachings herein, significant plasma levels (C_{max}) of interferon-β are achieved following intranasal administration of a pharmaceutical formulation of interferon-β in combination with one or more permeabilizing peptides of JAM, claudin, or occludin. The time to maximum serum concentration (t_{max}) by intranasal delivery will be accelerated at least approximately 5- to 10-fold, often sufficient to achieve similar blood plasma levels (C_{max}) when compared to subcutaneous or intramuscular injection. The rate of delivery of interferon-β by intranasal administration of pharmaceutical formulations of the present invention (as measured by C_{max} and t_{max}) is significantly increased when compared to the rate of delivery by intramuscular or subcutaneous injection of interferon-β.

The potential to deliver and maintain consistent therapeutic blood levels and CNS levels of interferon-β by pharmaceutical formulations of the present invention provide a distinct advantage over existing formulations for intramuscular or subcutaneous administration. A distinct advantage exists for maintaining consistent therapeutic blood levels and CNS levels of interferon-β by repeated intranasal administration within a 0.5 to 1 hour time frame in which maximum concentration in the blood serum is achieved, as compared to subcutaneous administration which requires 4 hours or longer to reach maximum concentration in the blood serum. Pharmacodynamic markers of interferon-β activity indicate a maximum concentration of IFN-β markers, neopterin and β_2-microglobulin, are achieved in 30 hours or less, or typically 22 hours or less following intranasal administration of interferon-β by pharmaceutical formulations of the present invention.

TABLE 14: Pharmacokinetic and pharmacodynamic parameters[a]

	Rebif®, SC 12 MIU (60 µg) dose	Rebif®, IM 12 MIU (60 µg) dose	Avonex®, IM 12 MIU (60 µg) dose	Intranasal Formulation F9 12 MIU (60 µg) dose	Intranasal Formulation JAM-1 NP-A + F9 (12 MIU) dose	Intranasal Formulatio n CLAUDIN 2 NP-27 + F9 (12 MIU) dose	Intranasal Formulatio n OCCLUDI N NP-26 + F9 (12 MIU) dose
Serum IFN-β:							
$AUC_{0\text{-}24h}$ (IU h/ml)	65	70	65	70	85	78	75
C_{max} (IU/ml)	5.1	5.2	4.9	4.4	6.0	5.6	4.5
t_{max} (h)	3	3.5	4	0.4	0.3	0.3	0.4
Serum neopterin:							
$AUC_{0\text{-}144h}$ (nmol h/l)	2700	2930	2974	195.5 (0-96h) ng•h/ml	263 (0-96h) ng•h/ml	243 (0-96h) ng•h/ml	196 (0-96h) ng•h/ml
C_{max} (nmol/l)	32	35	36	2.82 ng/ml	3.86 ng/ml	3.54 ng/ml	2.90 ng/ml
t_{max} (h)	36	36	36	23.9	19.1	19.1	23.9
Serum β_2-microglobulin:							
$AUC_{0\text{-}24h}$ (mg h/l)	271	277	270	238 (0-96h) µIU•h/ml	329 (0-96h) µIU•h/ml	305 (0-96h) µIU•h/ml	246 (0-96h) µIU•h/ml
C_{max} (mg/l)	2.3	2.4	2.3	2.1 µg/ml	2.8 µg/ml	2.6 µg/ml	2.1 µg/ml
t_{max} (h)	24	36	36	35.3	26.5	30.9	34.3

[a]Per hour and 10^4 cells.
*P= 0.015, Avonex® IM > Rebif® SC

Data on Avonex® and Rebif®: Munafo, et al., Eur. J. Neurology, 5: 187-193, 1998, .

In accordance with the foregoing teachings Table 14, below, provides projected exemplary pharmacokinetic for intranasal delivery of interferon-β-1a in a pharmaceutical formulation of the present invention (e.g., Formulation F-9 plus permeabilizing peptides of JAM-1, claudin-2, or occludin) compared to subcutaneous or intramuscular delivery of interferon-β-1a (Avonex® or Rebif®) or intranasal adminstration of Formulation F-9. The projected results in Table 15 compare simultaneous intranasal delivery of interferon-β-1a and permeabilizing peptides, JAM-1, claudin-2, or occludin, compared to intranasal delivery of permeabilizing peptides, JAM-1, claudin-2, or occludin preceding intranasal delivery of interferon-β-1a by 10, 20 or 30 minutes.

Maximum concentration of interferon-β in the blood serum (C_{max}) at 3 hours post dosing is projected to be approximately 6.0 IU/mL for intranasal delivery of Formulation F-9 (IFN-β-1a) plus permeabilizing peptide JAM-1 NP-A administered simultaneously. C_{max} is projected to be approximately 6.1 IU/mL for intranasal delivery of permeabilizing peptide JAM-1 NP-A preceding by 10 minutes intranasal delivery of Formulation F-9, or approximately 6.3 IU/mL for intranasal delivery of permeabilizing peptide JAM-1 NP-A preceding by 20 minutes or 30 minutes intranasal delivery of Formulation F-9. These values compare to 5.1 IU/mL for subcutaneous delivery of interferon-β-1a (at 12 MIU dose) or 4.9 to 5.2 IU/mL for intramuscular delivery of interferon-β-1a (at 12 MIU dose).

Time to maximum serum concentration of interferon-β in the blood serum (t_{max}) is projected to be approximately 0.3 hours for intranasal delivery of Formulation F-9 (IFN-β-1a) plus permeabilizing peptide JAM-1 NP-A administered simultaneously. t_{max} is projected to be approximately 0.25 hours for intranasal delivery of permeabilizing peptide JAM-1 NP-A preceding by 10 minutes intranasal delivery of Formulation F-9, or approximately 0.2 hours for intranasal delivery of permeabilizing peptide JAM-1 NP-A preceding by 20 minutes or 30 minutes intranasal delivery of Formulation F-9. These values compare to a t_{max} of 3 to 4 hours for intramuscular or subcutaneous administration of Avonex® or Rebif®.

As measured by pharmacodynamic IFN-β markers, neopterin and β_2-microglobulin, intranasal administration of a pharmaceutical formulation comprising permeabilizing peptide of the present invention, e.g., JAM, claudin, or occludin peptide, 10 minutes, 20 minutes or 30 minutes prior to intranasal administration of

interferon-β formulation provides advantages to improve delivery of interferon-β to the CNS or blood serum by 5 to 10 percent, 10 to 15 percent, or 15 to 20 percent compared to intranasal administration of interferon-β formulation F9 alone.

TABLE 15: Pharmacokinetic and pharmacodynamic parameters for intranasal administration of permeabilizing peptide, 10 minutes, 20 minutes or 30 minutes prior to intranasal administration of interferon-β formulation

	Intranasal Formulation F9 12 MIU (60 μg) dose	Intranasal Formulation F9 (12 MIU) + JAM-1 NP-A Simultaneous dose	Intranasal Formulation F9 (12 MIU) + JAM-1 NP-A at 10' preceding dose	Intranasal Formulation F9 (12 MIU) + JAM-1 NP-A at 20' preceding dose	Intranasal Formulation F9 (12 MIU) + JAM-1 NP-A at 30' preceding dose
Serum IFN-*β*:					
$AUC_{0\text{-}24h}$ (IU•h/ml)	70	85	80	85	80
C_{max} (IU/ml)	4.4	6.0	6.1	6.3	6.3
t_{max} (h)	0.4	0.3	0.25	0.20	0.20
Serum neopterin:					
$AUC_{0\text{-}96h}$ (ng•h/ml)	161.7	195.5	184.5	196.5	185.4
C_{max} (ng/ml)	2.07	2.82	2.87	2.96	2.99
t_{max} (h)	28.7	23.9	23.7	22.5	22.1
Serum β_2-microglobulin:					
$AUC_{0\text{-}96h}$ (μIU•h/ml)	197.5	238	225	239	226
C_{max} (μg/ml)	1.74	2.12	1.98	2.11	2.01
t_{max} (h)	41.7	35.3	33.4	31.3	29.8

Although the foregoing invention has been described in detail by way of example for purposes of clarity of understanding, it will be apparent to the artisan that certain changes and modifications are comprehended by the disclosure and may be practiced without undue experimentation within the scope of the appended claims, which are presented by way of illustration not limitation.